# The pathogenesis of endometriosis : Sampson was right

# The Pathogenesis Of Endometriosis

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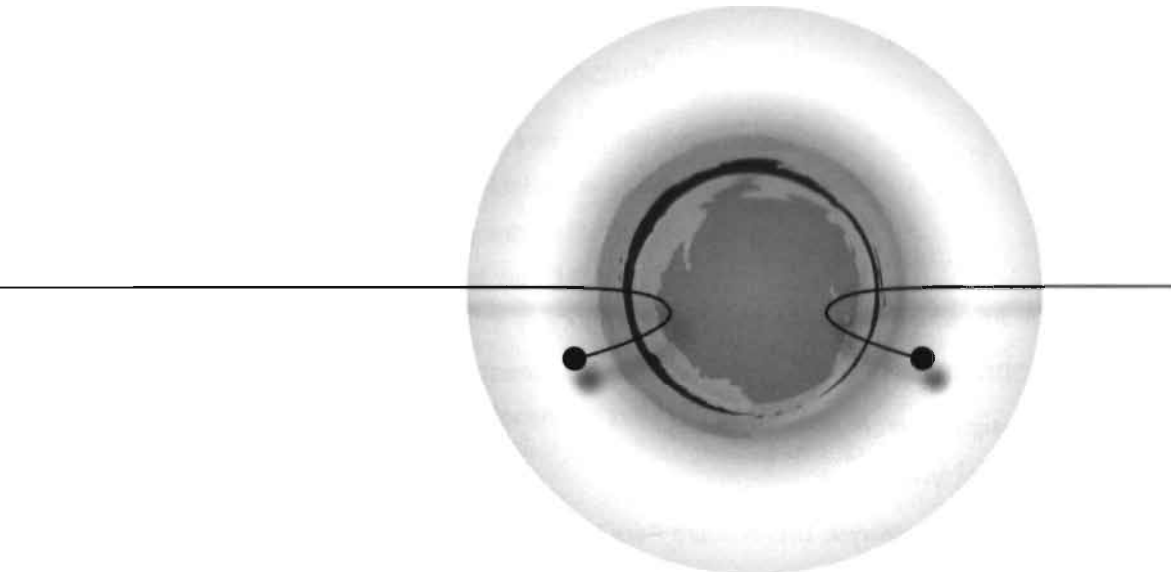

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# THE PATHOGENESIS OF ENDOMETRIOSIS

## SAMPSON WAS RIGHT

Proefschrift

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It is not a disaster to be unable to capture your ideal,
but it is a disaster to have no ideal to capture.
It is not a disgrace not to reach the stars,
but it is a disgrace to have no stars to reach for.
Not failure, but low aim is sin.

*Origin unknown*

*Voor Peter en Jasper*

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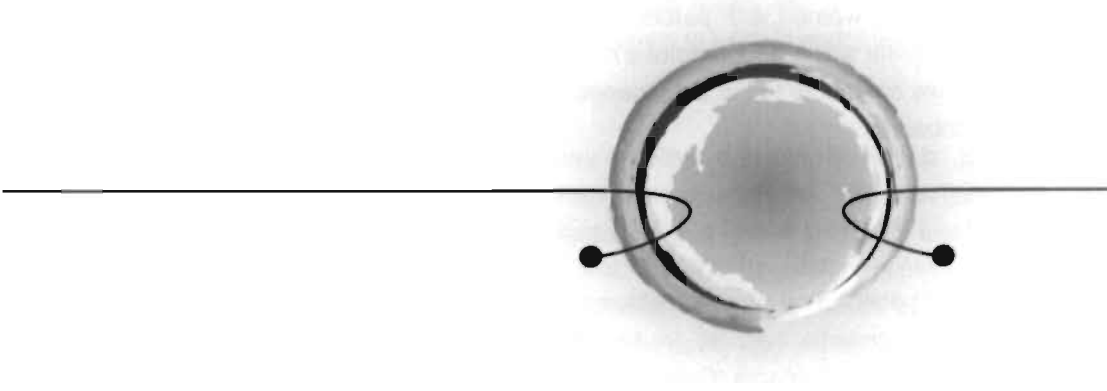

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## 1.1 Introduction

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterine cavity. Women with endometriosis present with characteristic signs and symptoms as dysmenorrhoea, dyspareunia, chronic pelvic pain or subfertility. Endometriosis is one of the most commonly encountered benign problems in gynaecology. It develops predominantly in women during the reproductive age and regresses after menopause or ovariectomy. A growing body of evidence indicates that a combination of genetic, hormonal, immunologic and anatomic factors contribute to the formation and development of the ectopic foci of endometrium. The natural history of peritoneal endometriosis is not exactly known, but most data support the contention that endometriosis in its superficial form is generally self-limiting. The notion of two distinct clinical entities, endometriosis as a phenomenon occurring intermittently in all menstruating women with patent tubes, and endometriosis as a disabling disease occurring in a subset of women, regained support in recent years (Koninckx, 1994). This concept, however, is not new: already in 1953 the idea of intermittent occurrence of superficial endometriosis was thus worded by Scott: "If serial section of all pelvic tissue were feasible might not all 40 year old women with patent tubes and normal menstrual cycles regardless of parity reveal some endometriosis?" (Scott *et al.*, 1953). In this chapter, the events that may contribute to the pathogenesis of the peritoneal endometriotic lesion will be described.



## 1.2 Pathogenesis

The first description of endometriosis was given by Cullen in 1896, who suggested that adenomyotic nodules in the rectovaginal septum resembled the mucous membrane of the uterus (Cullen, 1896). Later on, endometriosis was also described as implants of endometrium-like tissue on the peritoneum and the ovary. Because of the different locations, possible origins, appearances and hormone responsiveness, it was suggested recently that peritoneal endometriosis, ovarian endometriosis and adenomyotic nodules of the rectovaginal septum or deep invasive endometriosis are three different entities (Nisolle and Donnez, 1997), each with a different pathogenesis. It was argued, however, that deep invasive endometriosis does not originate in the rectovaginal septum, but develops from superficial endometriotic implants in the pouch of Douglas (Vercellini *et al.*, 2000). The most viable theses nowadays are the induction theory, the in-situ development theory and the retrograde transplantation theory.

### 1.2.1 Induction theory

The proponents of this theory assume that degenerating menstrual endometrium releases endogenous factors, which subsequently induce a metaplastic process in the serosal epithelium of the ovaries and in the serosal cells of the mesothelium, resulting in endometrial tissue (Levander and Normann, 1955; Merill, 1966; Ohtake *et al.*,1999). To meet the criteria for the definition of endometriosis both endometrial glands and stroma should be present in the ectopic lesion. The reports supporting this theory have provided evidence that endometrium-like epithelium and glands are formed as a result of induction. However, no direct evidence showing the formation of endometrial stroma has been reported at the end of the metaplastic process.

### 1.2.2 In-situ development theory

According to this theory, ectopic endometrium develops in situ from local tissues, including germinal epithelium of the ovary and remnants of the Müllerian and Wolffian ducts. In a broader context this theory also implies that peritoneal endometriosis is a result of in situ metaplasia of mesothelial serosal cells, which are totipotential (Haney, 1990; Fuji, 1991; Suginami, 1991). The fact that endometriosis mostly occurs when endometrium is present and that males are spared from this disease, weakens the power of the concept metaplasia to explain endometriosis.

### 1.2.3 Retrograde transplantation theory

Sampson's retrograde transplantation theory implies that endometriosis is a consequence of the reflux of endometrial fragments through the Fallopian tubes during menstruation, with subsequent implantation and growth on and into the peritoneum and the ovary (Sampson, 1921; 1927; 1940). The reflux implantation theory is based on the assumption that retrograde menstruation takes place and that viable endometrial tissue reaches the abdominal cavity and implants. Sampson based his theory largely on clinical and anatomical observations, but over the years experimental evidence has been provided supporting his hypothesis. Retrograde menstruation is a common event in women with patent Fallopian tubes. Halme and co-workers obtained peritoneal fluid by laparoscopy in the perimenstrual period. Blood was found in 90% of the patients with patent tubes. If the Fallopian tubes were occluded only 15% had evidence of blood in the pelvis (Halme *et al.*, 1984). Sampson's theory is also supported by the demonstration of the viability of shed menstrual endometrium in tissue culture (Halme *et al.*,1984; Koks *et al.*,1997), the distribution of endometriotic lesions in the abdominal cavity (Jenkins *et al.*, 1968), the high prevalence of pelvic endometriosis in girls with congenital menstrual outflow obstruction (Sanfilippo *et al.*, 1987) and animal experiments in which endometriosis was induced by the creation of uteropelvic fistulas (Te Linde *et al.*,

1950) or by obstruction of antegrade menstruation, thus forcing retrograde menstruation to take place (D'Hooghe *et al.*, 1994). The latter observations suggest that increased retrograde shedding of menstrual endometrium increases the likelihood of developing endometriosis, which is supported by the finding that menstruations are often longer and heavier in women with endometriosis (Darrow *et al.*, 1993; Vercellini *et al.*,1997).

## 1.3 Early endometriotic lesion formation

### 1.3.1 Evading the defence mechanisms in the abdominal cavity

Haney and co-workers were the first to report that menstrual effluent evokes an inflammatory response when arriving in the abdominal cavity (Haney *et al.*, 1981). It attracts large numbers of polymorph nuclear neutrophils (PMNs), and subsequently phagocytic and chemotactic leukocytes from the circulation (Haney *et al.*, 1981; Hill *et al.*, 1988).

Prior to the onset of menstruation a marked influx of bone marrow-derived cells is observed. Of these cells approximately 70% are CD56+ natural killer (NK) cells, 20% CD14+ macrophages and 10% CD3+ T-cells (Jones *et al.*, 1998). Conceivably, the regurgitated menstrual effluent also contributes to the increased numbers of endometrial cells in the peritoneal cavity.



The physiological role of the inflammatory response is to clear the ectopic cells and tissue from the abdomen. This is apparently not a very effective system since microscopic endometriosis is probably intermittently present in all women with patent Fallopian tubes and menstrual cycles (Koninckx, 1994). Longer menstrual periods and heavier menstrual blood flow will result in larger amounts of endometrial tissue in the abdominal cavity, which increases the risk of developing symptomatic endometriosis. Larger tissue fragments may have the capacity to develop into endometriotic lesions. They may have a higher chance of survival, since cells residing inside may be protected from the proteolytic enzymes and phagocytotic activity. These cells continue to produce angiogenic factors as a result of the continued hypoxic conditions. The eutopic endometrium of women with endometriosis was also shown to be more resistant to lysis by NK cells than the eutopic endometrium of controls (Oosterlynck *et al.*, 1991), suggesting that endometrial tissue from endometriosis patients resides in the abdominal cavity for a longer period of time.

Evidence is available now to support the notion that protein factors present in the peritoneal fluid are able to affect processes in the peritoneum. Dunselman and co-workers demonstrated that small proteins with a molecular weight (MW) <40 kD

could readily be exchanged between the vasculature and the peritoneal fluid (Dunselman *et al.*, 1988). Therefore, if the concentration of a certain factor is elevated in the peritoneal fluid, it may reach the circulation. It was shown that upon intraperitoneal injection, interleukin (IL)-4 was able to inhibit basic Fibroblast Growth Factor (bFGF)-induced corneal neovascularisation (Volpert *et al.*, 1998). Also, mice bearing tumours in the flank, and receiving repeated intraperitoneal injections of an antibody against macrophage inhibitory factor (MIF), showed significantly reduced tumour growth (Ogawa *et al.*, 2000). This suggests that factors present in the peritoneal fluid, which is basically exsudate from the peripheral blood, can also enter or re-enter the circulation and exert their influence on endometriotic lesions in a systemic way.

### 1.3.2 Adhesion to the peritoneal surface

#### *1.3.2.1 Mesothelium*

Biopsied proliferative and secretory endometrial fragments, as well as antegradely shed menstrual endometrial fragments only adhere at locations where the mesothelial lining of the peritoneum is damaged, exposing the basement membrane and/or the extracellular matrix (ECM) (Groothuis *et al.*, 1999; Koks *et al.*, 1999). This suggests that an intact mesothelial lining serves as a barrier and prevents adhesion of menstrual endometrial fragments to the peritoneal lining, thus preventing the implantation of endometrial tissue (Dunselman *et al.*, 2001). However, the mesothelium is fragile and can easily be damaged by surgery, inflammatory cells, or endometrium, thereby facilitating adhesion of endometrium. Analogous to tumour cells and tumour ascites, it was shown that cells isolated from menstrual effluent as well as conditioned medium prepared from menstrual effluent are able to induce morphological alterations in mesothelial cells (Koks *et al.*, 2000; Demir Weusten *et al.*, 2000). Therefore, menstrual endometrium is harmful to the mesothelium and may create its own adhesion sites at the mesothelial lining, thereby facilitating the development of endometriosis (Demir Weusten *et al.*, 2000). The morphological alterations are in fact epithelial-mesenchymal transitions (Demir *et al.*, 2003).



In other studies, in which single endometrial cells or glands were used, it was demonstrated that endometrial stromal and epithelial cells can adhere to intact mesothelium. This process would be mediated by the interaction between CD44 which is expressed on endometrial cells, and hyaluronic acid which is abundantly present on mesothelial cells. Individual endometrial (stromal) cells could migrate across a mesothelial cell monolayer within 18-24 hours (Witz *et al.*, 2001; 2002; Dechaud *et al.*, 2001).

*1.3.2.2 Cell adhesion molecules*

Adhesive properties of endometrial cells determine the process of attachment of retrogradely shed endometrium to the peritoneal lining. Several cell adhesion molecules are expressed by endometrial cells and modulate cell-matrix and cell-cell attachment, including integrins, cadherins, laminin-binding proteins, the immunoglobulin superfamily and CD44 (Stetler-Stevenson *et al.*, 1993). In endometrium and endometriosis, integrins and cadherins have been studied extensively. The integrins belong to a large family of transmembrane glycoproteins that provide an anchorage for cells to the ECM and are involved in direct invasion and motility of cells (Curran and Murray, 2000). The expression of integrins in the endometrium changes during the menstrual cycle (Tabibzadeh 1992; Van der Linden *et al.*, 1995; Lessey *et al.*, 1998). As conflicting results have been reported with regard to integrins in endometrium and endometriosis, their exact role remains controversial, but aberrant patterns of integrin expression have been associated with reproductive problems, including endometriosis (Lessey *et al.*, 1994; Starzinski-Powitz *et al.*, 1999).

Cadherins are transmembrane glycoproteins which mediate cell-cell adhesion. They can act as invasion suppressor molecules by inhibiting the ability of cells to escape from their primary site to initiate invasion. Immunohistochemical studies demonstrated that epithelial cadherin (E-cadherin) is expressed in epithelial glandular cells derived from menstrual effluent, in endometrium throughout the menstrual cycle, in peritoneal fluid, in peritoneum and in endometriosis, indicating a role in the maintenance of the epithelial architecture in endometrium (Van der Linden *et al.*, 1994; 1995). *In vitro*, E-cadherin positive cells are retained in tumour tissue by cell-cell interaction, but when E-cadherin is absent or inactivated, these cells are no longer constrained and invasion can occur (Guilford *et al.*, 1999). E-cadherin is therefore considered one of the central players in the establishment of metastasis in human carcinomas (Starzinski-Powitz *et al.*, 1999). Gaetje and Starzinski-Powitz hypothesize that endometriotic cells and carcinoma cells share molecular mechanisms of invasion and metastasis that are related to the absence of E-cadherin (Gaetje *et al.*, 1997; Starzinski-Powitz *et al.*, 1999).



### 1.3.3 Invasion

Early lesion formation is an invasive event, which requires ECM breakdown. The ECM consists of collagens, proteoglycans and glycoproteins including fibronectin and laminin. In addition to its role in determining cell shape, the ECM is important in metabolic processes, influencing cellular proliferation, differentiation and apoptosis, and it serves as a repository for biologically active growth factors. Remodelling and breakdown of the ECM is mainly regulated by matrix metalloproteinases (MMPs).

The involvement of MMPs in these processes was suspected after finding collagen breakdown products in the peritoneal fluid of endometriosis patients with mild endometriosis (Spuijbroek *et al.*, 1992). Later, intervention studies demonstrated that lesion formation can be prevented in mice with experimentally induced endometriosis, by inhibiting MMP production and activation (Bruner *et al.*, 1997).

MMPs are a family of structurally related, zinc-containing endoproteases that share structural domains but differ in substrate specificity, cellular sources and inducibility. All MMPs share the following functional features: they are capable of degrading ECM components, they are secreted in a latent proform and require activation for proteolytic activity, they contain zinc at their active site and need calcium for stability, they function at a neutral pH, and they are inhibited by specific inhibitors of metalloproteinases (TIMPs) (Creemers *et al.*, 2001). According to their substrate specificity and structure, members of the MMP family can be classified into subclasses: the collagenases, gelatinases, stromelysins, membrane-type (MT-) MMPs, and other MMPs.

The activity of MMPs is controlled by the induction of gene expression and by the activation of latent pro-enzymes. Induction at the level of gene expression is mediated by growth factors, hormones, and inflammatory cytokines including IL-1, IL-6, tumour necrosis factor-α (TNF-α), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) (Malik *et al.*, 1996; Schonbeck *et al.*, 1997). The activation of the latent pro-enzymes can be achieved (1) by stepwise activation in which plasmin is assumed to be the most potent physiological activator *in vivo*, (2) by activation at the cell-surface by MT-MMPs, and (3) by intracellular activation (Murphy *et al.*, 1994; Nagase, 1997). In turn, the activity of MMPs in tissues is controlled by the antagonizing actions of their natural inhibitors, the TIMPs. TIMPs are present in the majority of tissues and body fluids, and are expressed by a variety of cells. TIMP-1, -2, -3 and -4 are structurally related and bind noncovalently to active MMPs. They have the ability to interact with the zinc-binding site within the catalytic domain of active MMPs.



In many reproductive processes including menstruation, ovulation, and embryo implantation MMPs are expressed in a highly regulated manner (Marbaix *et al.*, 1995; Hulboy *et al.*, 1997). In the endometrium, MMP expression is most pronounced during menstruation. A limited number of MMPs is expressed at low levels during the proliferative phase of the menstrual cycle, whereas expression generally declines during the early secretory phase, and reappears in the late secretory phase. Endometrial MMP expression is regulated by hormones, cytokines and growth factors. In particular, progesterone is a potent repressor both *in vitro* (Rodgers *et al.*, 1994; Bruner *et al.*, 1999) and *in vivo* (Spuijbroek *et al.*, 1992). The

mechanism through which progesterone modulates MMP and TIMP activity is not yet clear. It has been proposed that progesterone regulates MMP expression indirectly through controlling the plasminogen activator pathway. Progesterone can increase the levels of plasminogen activator inhibitor (PAI)-1 and thus reduce plasmin-mediated activation of latent MMPs (Clark, 1992). Locally produced retinoic acid and transforming growth factor-β (TGF-β) were shown to act as mediators of the progesterone suppression of MMPs, while enhancing expression of TIMPs (Osteen *et al.*, 2002). On the other hand, arguments against progesterone as the primary regulator of endometrial collagenase activity are that *in vivo* circulating progesterone levels decrease too early to explain the peri-menstrual increase in MMP expression, and tissue degradation at menstruation occurs at focal points rather than throughout the entire endometrium (Salamonsen and Woolley, 1996).

A number of MMPs has been described to be associated with endometriosis (reviewed by Osteen *et al.*, 1996, 1999; Sillem *et al.*, 1998; and Sharpe-Timms and Cox, 2002). The drawback of most studies available until now with regard to the role of MMPs in endometriosis is that they are only descriptive at the level of MMP expression, and that the functional involvement of MMPs in the development and progression of endometriosis has not been proven. Expression of a selection of MMPs has been reported, either because only a limited number of MMPs has been studied, or because expression of certain MMPs has not been found. In order to gain more insight in the functional role of MMPs in endometriosis, more intervention studies are required.



### 1.3.4 Acquisition of a blood supply

Ectopically implanted endometrium needs to trigger an angiogenic response, activating angiogenesis within and around the tissue, in order to survive in its new environment. Angiogenesis is the formation of new blood vessels from pre-existing capillaries. It involves the proteolytic degradation of the ECM, proliferation and migration of endothelial cells, and ultimately formation of a patent tubular network supplying the angiogenic stimulus.

Angiogenesis is essential in growth, wound healing, and in the female reproductive system including processes in the menstrual cycle and pregnancy. It occurs when the balance of local factors favoring vascular growth exceeds those factors inhibiting angiogenesis. Disruption of the balance between inhibitors and activators in favour of excessive angiogenesis may result in conditions such as cancer, atherosclerosis, chronic inflammation and endometriosis (McLaren, 2000). Several factors of peptide and non-peptide nature induce angiogenesis *in vivo* (reviewed by Smith, 1998). Vascular endothelial growth factor (VEGF) is suggested to be the most important factor, based on its ability to induce endothelial cell proliferation and migration, to

induce vasodilatation, and on its endothelial cell permeability increasing effect (Griffioen and Molema, 2000).

Studies on the vascularisation of endometriotic lesions are rare, despite the fact that at laparoscopy, active superficial endometriotic lesions are easily recognized by the abundant vasculature. Two studies have compared the vascular density and luminal diameter in red, black, and white lesions as well as in endometriomas and lesions in the rectovaginal septum. Donnez and co-workers described that red lesions were better vascularised, and that this was the result of a larger vessel diameter rather than the number of vessels, which was significantly higher in black lesions (Donnez *et al.*, 1998). The group of Matsuzaki found no differences in the numbers of vessels between the different types of lesions, but described that red lesions had more vessels with a small diameter (<10 μm), whereas black lesions had significantly more vessels with a larger diameter (>20 μm) (Matsuzaki *et al.*, 2001). The fact that endothelial cell proliferation and smooth muscle cell-negative bloodvessels were observed in these lesions, suggests that an angiogenic process is ongoing in these lesions.

Studies in the chicken chorioallantoic membrane (CAM) model indicate that human endometrium is highly angiogenic, and therefore capable of attracting blood vessels from the surrounding tissue (Maas *et al.*, 1999). In the endometrium, angiogenesis is regulated by many angiogenic factors, of which VEGF-A appears to be the most important one. In the proliferative phase of the menstrual cycle, oestradiol triggers VEGF-A expression in endometrial epithelial cells and stromal fibroblasts, which is increased in the secretory phase (Charnock-Jones *et al.*, 1993). Prior to menstruation, the endometrium becomes hypoxic as a result of vasoconstriction, which enhances the production of VEGF-A in the endometrial tissue even more (Smith, 2001).



In eutopic endometrium of endometriosis patients endothelial cell proliferation and VEGF-A content were significantly higher than in the endometrium of disease-free women (Donnez *et al.*, 1998; Wingfield *et al.*, 1995). The significance of VEGF-A and of angiogenesis in early endometriotic lesion formation was illustrated by Hull and co-workers (Hull *et al.*, 2003). Antagonizing the actions of VEGF-A by administering the soluble VEGF receptor sflt-1 reduced the number of lesions formed in nude mice after intraperitoneal injection of minced endometrial tissue. This suggests that anti-angiogenic therapy may be effective in preventing the development of endometriosis.

### 1.3.5 Survival post-implantation

Endometriosis is an estrogen dependent disease. Hormonal treatment of endometriosis, with for instance Gonadotrophin Releasing Hormone (GnRH) analogues or progestins, is aimed at creating a hypoestrogenic environment, which keeps the endometrium dormant. An increase in proliferative activity of endometrial cells has been demonstrated in the eutopic endometrium of women with endometriosis as compared to the eutopic endometrium of women without the disease (Wingfield *et al.*, 1995; Jurgensen *et al.*, 1996). However, the relevance of this observation is disputed by the finding that hardly any difference in proliferation was present between eutopic and ectopic endometrium (Nisolle *et al.*, 1997), or even a reduced proliferation in the epithelium of endometriotic lesions compared to eutopic endometrium (Scotti *et al.*, 2000). This supports the contention that the implantation of endometrium is a process which requires extensive changes in tissue organization, rather than increased proliferation.

It has become evident that local production of estrogens is an important factor in the pathogenesis of diseases of the female reproductive tract. This is also the case in endometriosis. The conversion of estrogens from androgens is catalysed by aromatase P450. Aromatase is normally expressed in a number of human tissues including the ovary and the adipose tissue, but usually not in normal endometrium (Noble *et al.*, 1996; Kitawaki *et al.*, 1997). However, this enzyme has been found in endometriotic tissue and in the endometrium of women with endometriosis (Noble *et al.*, 1995; 1997). Moreover, endometriotic tissue is often deficient in 17β-hydroxysteroid dehydrogenase (17β-HSD) type 2, which normally converts the strong estrogenic 17β-oestradiol into the weak estrogenic estrone. Consequently, this protective mechanism that lowers oestradiol levels is lost in endometriotic tissue (Zeitoun *et al.*, 1998). The higher oestradiol level of menstrual effluent in women with endometriosis as compared to controls supports this hypothesis (Takahashi *et al.*, 1989).



As a result of increased estrogen production in eutopic and ectopic endometrium, a positive feedback loop is initiated, which results in the continuous local production of estrogen (Noble *et al.*, 1997; Bulun *et al.*, 2001). Estrogen stimulates the production of cyclooxygenase type 2 (COX-2) enzyme. This results in elevated levels of prostaglandin $E_2$ ($PGE_2$), which is a potent stimulator of aromatase activity in endometriosis.

The clinical relevance of these findings has been exemplified by the successfull treatment of an aggressive case of post-menopausal endometriosis using an aromatase inhibitor (Takayama *et al.*, 1998), and the beneficial effects of non-

steroidal anti-inflammatory drugs (NSAIDs) for the treatment of endometriosis-related pain symptoms (Corson *et al.*, 1978).

## 1.4 Problem

In order for endometrial tissue to implant in an ectopic location, it must be viable, adhere to the peritoneum, degrade the extracellular matrix, invade, acquire a blood supply, and survive. This sequence of events cannot be studied in man, but has been studied extensively in various model systems, *in vitro*, *ex vivo* and *in vivo*. From these studies it has become apparent that the endometrial tissue itself is a key element in the development of endometriosis. However, the exact role of endometrial tissue integrity, matrix metalloproteinases, angiogenesis and steroid hormones in the pathogenesis of the disease is unknown.

## 1.5 Hypothesis

Innate properties of human endometrium determine the success of implantation and endometriotic lesion formation.

## 1.6 Objectives

To test the hypothesis the following objectives were formulated.



1. To investigate the impact of tissue integrity on the success of implantation and of endometriosis-like lesion formation. To this end, endometrial tissue collected from all phases of the menstrual cycle was transplanted onto the CAM.
2. To study the involvement of MMPs in the early development of endometriotic lesions. The function of MMPs in endometriotic lesion formation was investigated by applying an MMP inhibitor to the CAM after transplantation of human endometrial fragments.
3. To determine whether angiogenesis is required for early endometriotic lesion formation. To this end, angiostatic agents were applied to the CAM after transplantation of human endometrial fragments.
4. To determine whether angiogenesis is required for the maintenance of already developed endometriotic lesions. To this end, angiostatic agents were administered intraperitoneally and subcutaneously to nude mice three weeks after lesion induction by transplantation of human endometrial fragments.

5. To study the influence of the use of oral contraceptives (OC) on the ability of endometrium to implant ectopically. To this end, menstrual endometrium and endometrium of OC users were compared with regard to the potential to form endometriosis-like lesions in the CAM. Microarray studies were performed to identify gene transcripts in menstrual endometrium and in endometrium of OC users that may be involved in the ectopic implantation of endometrium.

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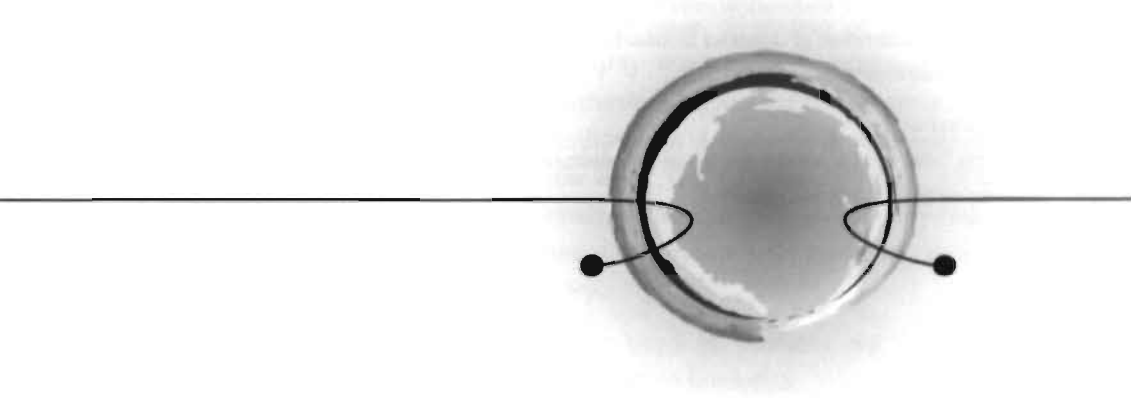
# Chapter 2

## Tissue integrity is essential for ectopic implantation of human endometrium in the chicken chorioallantoic membrane



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# Abstract

**Background**

Not all women with patent tubes develop clinically manifest endometriosis. Quality and quantity of endometrium in retrograde menstruation may be the determining factor in the development of the disease. We hypothesize that retrograde shedding of endometrial fragments with preserved integrity facilitates implantation of endometrium in ectopic locations, resulting in endometriotic lesion development. We evaluate the impact of tissue integrity on the success of endometriosis-like lesion development in the chicken embryo chorioallantoic membrane (CAM) model.

**Methods**

Menstrual and non-menstrual (cyclic) endometrium were collected by biopsy, and either minced or enzymatically dispersed. Spontaneously shed menstrual effluent was collected by a menstrual cup, and cells and tissue were isolated. We evaluated whether infiltration or lesion formation in the CAM occurred after transplantation of endometrium onto the CAM.

**Results**

Transplantation of biopsied menstrual and cyclic endometrial fragments, and of endometrial fragments larger than 1 $mm^3$ isolated from menstrual effluent resulted in lesion formation. Transplantation of endometrial cells isolated from menstrual effluent did not lead to lesion formation. After transplantation of digested biopsied cyclic endometrium, infiltration in the CAM but no lesions were observed.

**Conclusion**

In the CAM assay, integrity of tissue architecture determines the success of implantation of human endometrium in ectopic locations.

## Introduction

According to Sampson's hypothesis on the pathogenesis of endometriosis, viable, spontaneously shed endometrial tissue arrives in the abdominal cavity and implants in the peritoneum (Sampson, 1927). It is not clear how endometriotic lesions come into existence after the adhesion of endometrium to the peritoneum. Transplantation of endometrium, biopsied during the non-menstrual phase of the cycle (cyclic endometrium) results in the development of endometriosis-like lesions in the chicken embryo chorioallantoic membrane (CAM) that has been used to study tumour transplantation and invasion (Murphy, 1912; Leighton, 1964; Scher *et al.*, 1976). These endometriosis-like lesions consist of human stromal and glandular cells, as confirmed by in situ hybridisation for human chromosome 1 (Maas *et al.*, 2001).

Since menstrual endometrium enters the abdominal cavity and is supposedly "the seed" that develops into endometriotic lesions, menstrual endometrium is the most appropriate tissue to study the early pathogenesis of endometriosis (Groothuis, 1999). Until now, menstrual or endometrial characteristics responsible for the ectopic implantation of endometrium are not yet clear. Shedding of a sufficient amount of endometrium with preserved integrity may facilitate implantation of endometrium in an ectopic location. This hypothesis is supported by the association of increased amounts of retrograde menstruation with a higher risk of developing endometriosis (Sanfilippo *et al.*, 1986; Darrow *et al.*,1993; D'Hooghe *et al.*, 1994). Moreover, Sillem and co-workers demonstrated in the cynomolgus monkey model that collagenase digestion of endometrial tissue fragments prior to transplantation reduces the ability to implant (Sillem *et al.*, 1996).



In the present study, the CAM model will be used to evaluate the impact of endometrial tissue integrity on endometriosis-like lesion formation.

## Materials and methods

### Study design

We transplanted onto the CAM (1) biopsied cyclic endometrium, (2) biopsied menstrual endometrium, (3) cells and tissue fragments isolated from spontaneously shed menstrual effluent, and (4) enzymatically dispersed endometrium. To account for the effects of tissue handling and manipulation of endometrium, the impact of storage of endometrium in the Keeper (a vaginal cup used for the collection of spontaneously shed menstrual effluent) and of tissue handling on the potential to

form lesions was evaluated. To this end we transplanted onto the CAM (5) biopsied menstrual endometrium stored in the Keeper, and (6) biopsied menstrual endometrium stored in the Keeper and processed as under (3). Infiltration and endometriosis-like lesion formation were evaluated. In addition, proliferation in endometrium prior to and after transplantation onto the CAM was studied.

**Tissue handling**

*Biopsied endometrium*

Endometrium was collected by biopsy from 12 women with normal ovulatory cycles undergoing laparoscopy for benign conditions. An Endobiops endometrium sampling device (Gynotec, Malden, The Netherlands) was used. Endometrium was collected during the non-menstrual phase of the cycle (n=6, cyclic endometrium) or during menstruation (n=6, menstrual endometrium). After collection, endometrium was placed in serum-free medium composed of DMEM/HAM's F12 supplemented with 2 mmol/l L-glutamine (Gibco Life Technologies, Breda, The Netherlands), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B, and stripped of blood. The endometrial tissue was carefully dissected into uniform pieces of 1-2 $mm^3$ and transplanted onto the CAM (Maas *et al.*, 2001). To monitor infiltration and lesion development *after transplantation of biopsied menstrual* endometrium, CAMs were embedded in paraffin after 24, 48 and 72 hours.

*Isolation of endometrium from menstrual effluent*



Menstrual effluent was collected by 10 volunteers with regular menstrual cycles. These women had no history of endometriosis and did not use oral contraceptives. Collection took place in a menstrual cup for 2 to 3 hours during day 1 or day 2 of menstruation. The soft natural rubber cup, shaped like a cone ("Keeper", Den Haag, The Netherlands), was inserted into the upper vagina with the opening enclosing the cervix. Immediately after collection, the menstrual effluent was brought to the laboratory in a sterile plastic container (Koks *et al.*, 1997). Menstrual effluent was resuspended in serum-free medium, layered on a Ficoll-Paque gradient (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and centrifuged at 1200 *g* for 30 minutes at 4°C. The interphase containing endometrial and inflammatory cells was collected and washed twice in routine medium. The pellet was resuspended in 20-30 μl of serum-free medium and transferred onto the CAM.

In case endometrial fragments larger than 1 $mm^3$ could be identified in the menstrual effluent just below the interphase after the Ficoll-Paque gradient centrifugation, these fragments were picked up from the suspension with a forceps and transplanted onto the CAM.

*Collagenase digestion of cyclic endometrium*

In order to evaluate the effect of destruction of tissue integrity on the ability to form endometriosis-like lesions, collagenase digestion of endometrium was performed. After collection of biopsied cyclic endometrium (n=8), endometrium was rinsed in serum-free medium and minced into small pieces. The suspension was centrifuged, and the medium was replaced by medium containing 0.25% collagenase type I (ICN Biomedicals BV, Zoetermeer, The Netherlands) and 0.1% trypsin (Gibco BRL). The tissue was digested for 15 to 20 minutes at 37°C and filtered through a 400-μm stainless steel sieve (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). The cells were pelleted and washed once in serum-free medium. The pellet consisting of collagenase-digested glandular and stromal cells was resuspended in 20-30 μl of serum-free medium and transplanted onto the CAM.

*Biopsied menstrual endometrium stored in the Keeper*

To evaluate whether tissue collection in the Keeper affects the ability to form lesions, biopsied menstrual endometrium (n=4) was stored in a small amount of serum-free medium in a Keeper for 2 to 3 hours at 37°C. Subsequently, the endometrial tissue was stripped of blood and carefully sectioned into uniform pieces of 1-2 $mm^3$ and transplanted onto the CAM.

*Biopsied menstrual endometrium stored in the Keeper and processed as menstrual fluent*

To evaluate whether laboratory procedures necessary to isolate endometrial cells and tissue from spontaneously shed menstrual effluent affect lesion formation, biopsied menstrual endometrium (n=4) was kept in a Keeper at 37°C for 2 to 3 hours in a small amount of serum-free medium, and subsequently processed in the same way as menstrual effluent. After processing, endometrial tissue fragments were transplanted onto the CAM. Of each endometrial sample collected for this study, 1 tissue fragment was fixed in formalin immediately after collection and embedded in paraffin. Paraffin sections (4 μm) were cut and either stained with Haematoxylin and Eosin (H&E) for histological evaluation or stored for later immunohistochemical analysis. The use of human tissue for this study was approved by the Institutional Review Board of the University Hospital Maastricht, and all women participating in the study gave their written informed consent.



*CAM model*

Fertilised eggs of Lohman-selected White Leghorns were incubated for 3 days at 37°C, 55% relative air humidity, while being rotated hourly. At day 3 of incubation, a rectangular window (1 x 1.5 cm) was made in the eggshell. Two ml of albumen was

withdrawn using a 21 Gauge (G) needle, through the large blunt edge of the egg. The window was covered with Scotch tape to prevent dehydration. The eggs were replaced in the incubator without rotation until day 8 to 11 of incubation.

The CAM is an impenetrable barrier to invasive cells unless it has been traumatized by removing the upper peridermal part of the double epithelial layer, leaving the basal layer intact. Therefore, just before the transplantation of endometrium a small part of the CAM was gently traumatised by laying a 1 $cm^2$ wide strip of sterile ether-extracted lens paper onto the surface of the epithelium and then removing it immediately (Armstrong *et al.*, 1982; Maas *et al.*, 2001).

Endometrial tissue was transplanted onto the CAM. Following transplantation, the window was covered again and the egg was placed back in the incubator. After incubating for 24, 48, and 72 hours, the transplanted tissue including the surrounding CAM was excised, fixed in formalin and embedded in paraffin. Paraffin sections were cut and either stained with H&E for histological evaluation or stored for later immunohistochemical analysis.

### Immunohistochemistry

Proliferation was evaluated by immunohistochemistry using a mouse monoclonal antibody against the proliferation marker Ki67 (MIB-1, 1:25, Boehringer, Mannheim, Germany). Epithelium was stained using a mouse monoclonal antibody against pan-cytokeratin (Clone MNF 116, 1:100, DAKO, Glostrup, Denmark). In short, paraffin sections were deparaffinised by incubation with xylene for 2 times 3 minutes and rehydrated in alcohol series. Endogenous peroxidase activity was blocked by incubation with 0.25% hydrogen peroxide in methanol for 20 minutes. Sections were rinsed 3 times in phosphate-buffered saline (PBS) and were heated to 95°C in citrate buffer (pH 6.0) for 20 minutes in preparation for incubation with the MIB-1 antibody, or digested in 0.1% pepsin in 0.1N HCL for 30 minutes in preparation for incubation with the pan-cytokeratin antibody. After rinsing again in PBS, sections were incubated overnight at 4°C with the primary antibody. After 3 PBS rinses, sections were exposed to Envision anti-mouse for 30 minutes. After rinsing in PBS, antibody binding was visualized with 3'-3-diaminobenzidin (DAB). Sections were washed and counterstained with haematoxylin, washed again, dehydrated and mounted for light microscopy.



### Statistical analysis

Differences in infiltration and endometriosis-like lesion formation between biopsied cyclic endometrium and the other endometrial cell and tissue preparations used in this study were calculated using $\chi^2$ tests. P-values $<0.05$ were considered statistically significant.

## Results

### Lesion development in biopsied menstrual endometrium

Twenty four hours after transplantation of biopsied menstrual endometrium, endometrial cells were observed in direct contact with the CAM mesenchyme (Figure 1a, 1b). Forty eight hours after transplantation, lesions were observed consisting of endometrial glands and heterogeneous stroma, with blood vessels containing nucleated erythrocytes in close proximity of the edge of the lesions (Figure 1c). Seventy two hours after transplantation, organized lesions were observed in the CAM mesenchyme, with intact glands and surrounding endometrial stroma, mimicking normal endometrium and endometriotic lesions. Nucleated-erythrocytes containing vessels were present within the lesions (Figure 1d).

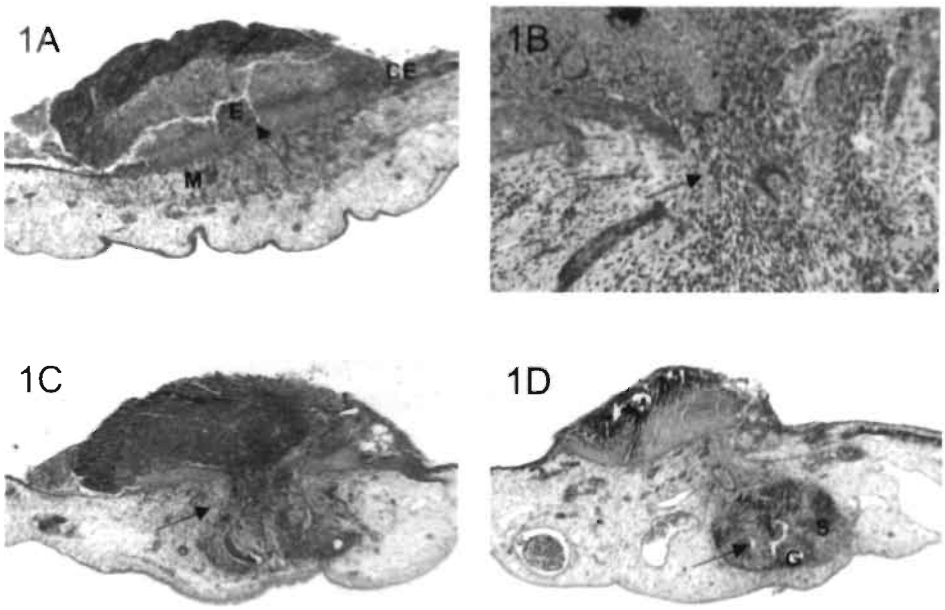




*Figure 1* *Development of endometriosis-like lesion after transplantation of biopsied menstrual endometrium onto the CAM.*
*(1A) Direct contact (arrow) of endometrium (E) and CAM mesenchyme (M) 24 hours after transplantation. CE: chorionic epithelium.*
*(1B) Detail of cytokeratin positive epithelial cells 24 hours after transplantation. Direct contact (arrow) of endometrium and CAM mesenchyme.*
*(1C) Infiltration (arrow) of endometrial tissue 48 hours after transplantation.*
*(1D) Organized lesion with intact glands (G) and surrounding endometrial stroma (S), with enlarged vessels in the CAM (arrow) 72 hours after transplantation. (A, C, D: haematoxylin and eosin staining, B: cytokeratin staining).*

### Proliferation

Prior to transplantation, Ki67 positive cells were present both in menstrual and in cyclic endometrium (Figure 2a). Seventy two hours after transplantation, Ki67 staining was almost absent (Figure 2b).

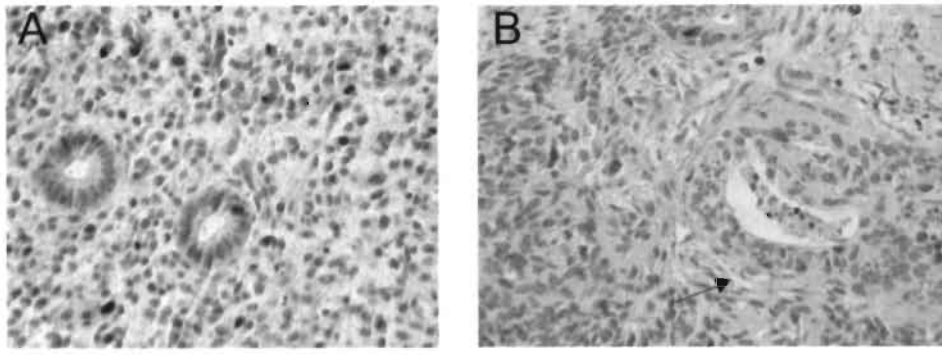


*Figure 2. Immunostaining of proliferation marker Ki67 in biopsied cyclic endometrium. (2A) Ki67 staining prior to transplantation, and (2B) 72 hours after transplantation. Note the vessels in the lesion (arrow).*

### Impact of tissue integrity on lesion formation

The abilities of the various cell and tissue preparations to infiltrate the CAM and to form endometriosis-like lesions 72 hours after transplantation are summarized in Table 1. Transplantation of biopsied cyclic and biopsied menstrual endometrium resulted in infiltration in 74% and 78% and in formation of endometriosis-like lesions in 68% and 67% of CAMs, respectively. Transplantation of endometrial cell suspensions isolated from spontaneously shed menstrual effluent collected by the Keeper resulted neither in infiltration nor in lesion formation in the CAM. In 4 out of 10 menstrual effluent samples, intact tissue fragments with a size larger than 1 $mm^3$ were present in menstrual effluent and could be picked up from the suspension with a forceps. These fragments were composed of intact glands and stromal tissue as confirmed by immunohistochemical staining for cytokeratin and vimentin respectively (data not shown). After transplantation onto the CAM they were able to infiltrate in 63% and to form lesions in 44% of CAMs. Transplantation of endometrium after collagenase digestion resulted in infiltration in 53%, but did not result in lesion formation in the CAM. After transplantation of biopsied menstrual endometrium that had been stored in the Keeper, or had been processed in the same way as menstrual effluent, infiltration as well as lesion formation in the CAM were still observed.

*Table 1. Impact of tissue integrity on infiltration and endometriosis-like lesion formation in the CAM*

| Endometrium | # of CAMs | Infiltration | Lesion |
|---|---|---|---|
| biopsied cyclic endometrium | 19 | 14 (74%) | 13 (68%) |
| biopsied menstrual endometrium | 18 | 14 (78%) | 12 (67%) |
| endometrial cells isolated from menstrual effluent collected in Keeper | 50 | 0 (0%) * | 0 (0%) * |
| tissue fragments in menstrual effluent collected in Keeper | 16 | 10 (63%) | 7 (44%) ** |
| collagenase digested biopsied cyclic endometrium | 45 | 24 (53%) ** | 1 (2%) * |
| biopsied menstrual endometrium, stored in Keeper | 13 | 10 (77%) | 9 (69%) |
| biopsied menstrual endometrium, stored in Keeper, processed as menstrual effluent | 11 | 6 (55%) | 4 (36%) ** |

*statistically significant compared to biopsied cyclic endometrium. *P<0.01; **P<0.1

## Discussion

### Lesion development

In this study we have shown that biopsied menstrual endometrium and endometrial tissue fragments isolated from spontaneously shed menstrual effluent are able to form endometriosis-like lesions in the CAM model, in a similar fashion as biopsied cyclic endometrium. Twenty four hours after transplantation, direct contact between the endometrium and the CAM mesenchyme is observed, and after 72 hours complete lesions are present in the CAM.



These observations imply that lesions originate from endometrial cells that have migrated into the CAM mesenchyme. Alternatively, lesions may develop from rapidly proliferating endometrial cells. Contrary to what we expected, proliferation marker Ki67 is hardly expressed in the lesions while it is expressed in the endometrium prior to transplantation. This suggests a minor role for cell proliferation in the organization of lesions. These findings are in accordance with results of other authors, who reported a significantly reduced proliferation activity in the epithelium of ectopic lesions as compared to the eutopic endometrium (Jones *et al.*,1995; Scotti *et al.*, 2000). Therefore, it is likely that the ability of infiltrated endometrial cells to rebuild the original tissue structure is responsible for lesion formation. However, we cannot exclude the possibility that hormonal or other environmental factors in the body which are absent in the CAM may play a role in lesion formation as well.

**Impact of tissue integrity on lesion formation**

Transplantation of biopsied cyclic and menstrual endometrium results in infiltration and endometriosis-like lesions, whereas transplantation of single endometrial cells isolated from spontaneously shed menstrual effluent does not. This suggests that the tissue architecture of biopsied endometrium, which consists of organized glandular epithelium and stroma, is pivotal for the ability of endometrium to infiltrate and to form lesions. During menstruation, endometrial tissue is exposed to high levels of matrix degrading enzymes (Koks *et al.*, 2000; Marbaix *et al.*, 1995). In most cases, menstrual effluent consists of single endometrial glandular and stromal cells instead of intact tissue fragments. These endometrial cells adhere easily to the basement membrane of amnion and peritoneum (Koks *et al.*, 1999), but they are not able to infiltrate and develop endometriosis-like lesions in the CAM. Apparently, single endometrial cells lack the essential mutual contact in which glandular structures are intact and surrounding stromal cells are present, and consequently lack the ability to form lesions. In 4 out of 10 menstrual effluent samples in this study, intact endometrial fragments larger than 1 $mm^3$ could be retrieved from the menstrual effluent. These fragments, composed of intact glandular structures and surrounding stromal cells, were able to induce endometriosis-like lesions in the CAM, whereas single cells from the same menstrual effluent samples were not.

Compared to biopsied endometrium, the number of lesions formed was slightly reduced, suggesting a diminished capacity of shed fragments of endometrium to form lesions, most likely as a result of the tissue degradation process which has been initiated at menstruation. Therefore, it is tempting to suggest that women who shed intact endometrial fragments in their menstrual effluent are more prone to develop endometriosis.



The capacity to form lesions disappeared once biopsied cyclic endometrium was digested by collagenase prior to transplantation. This is in accordance with the findings of Sillem and co-workers in the cynomolgus monkey model. These authors found that enzymatic treatment of endometrial tissue reduced the development of endometriotic lesions (Sillem *et al.*, 1996).

The observation that transplantation of endometrial cells isolated from menstrual effluent does not result in lesion formation may alternatively be a consequence of the contact of menstrual effluent with the Keeper, or of laboratory procedures necessary to isolate endometrial cells and fragments from menstrual effluent. For this reason we have exposed biopsied menstrual endometrium to storage in the Keeper, and to the same laboratory procedures that were used for isolation of endometrial cells and tissue from menstrual effluent. Lesion formation was not affected after storage of biopsied menstrual endometrium in the Keeper. When

biopsied menstrual endometrium was processed in the same way as shed menstrual effluent, lesion formation was slightly reduced. For this reason, we consider it safe to argue that laboratory procedures used to isolate endometrial cells and tissue from menstrual effluent prior to transplantation are not responsible for the complete lack of lesion formation after transplantation of cells isolated from menstrual effluent.

In conclusion, we have shown that biopsied menstrual endometrium is able to develop into endometriosis-like lesions in the CAM model. Spontaneously shed menstrual endometrium will also form lesions if tissue fragments are of sufficient size, assuring preservation of tissue architecture and interaction between epithelial glands and the stromal compartment. These findings indicate that tissue integrity is crucial for the development of endometriotic lesions.

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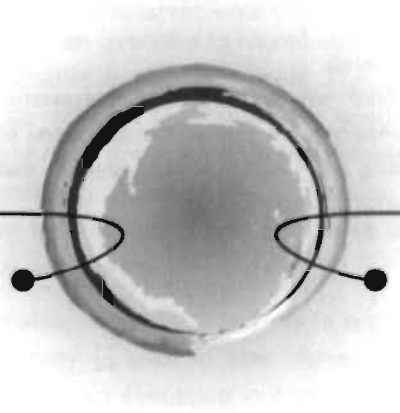
# Chapter 3

## Inhibiting MMP activity prevents the development of endometriosis in the chicken chorioallantoic membrane model



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# Abstract

**Background**

Matrix metalloproteinases (MMPs) are essential for extracellular matrix remodelling and may contribute to the development of endometriosis. Transplantation of endometrium onto the chicken chorioallantoic membrane (CAM) results in endometriosis-like lesion formation, a process that requires extensive tissue remodelling. We investigated the expression of a wide range of MMPs in menstrual endometrium, endometriosis-like lesions in CAMs, in peritoneal endometriosis and in endometriosis in the rectovaginal space, as well as the function of MMPs in early lesion formation in the CAM model.

**Methods**

Expression of MMPs was evaluated by immunohistochemistry and MMP function was studied in the CAM by inhibiting MMP activity during lesion formation.

**Results**

Nearly all MMPs were present in all tissues studied. No significant differences in the expression of a majority of MMPs were found in endometriosis-like lesions in CAMs when compared to human endometriosis. Inhibition of the MMP-1, -2, -3, -7 and -13 activities significantly impaired endometriosis-like lesion formation in CAMs.

**Conclusion**

The MMP expression profiles of experimentally induced endometriosis in CAMs and human endometriosis are similar. The prevention of endometriosis-like lesion formation in the CAM by inhibiting MMP activity strongly suggests that MMPs have a function in the early development of endometriotic lesions.

## Introduction

Endometriosis presents as lesions of endometrium with glands and stroma that grow at locations outside the uterus, predominantly within the peritoneal cavity. It presumably develops after the ectopic implantation of endometrium, which has entered the peritoneal cavity via the Fallopian tubes during menstruation (Sampson, 1927). The initial phase of the disease is an invasive event, which requires extracellular matrix (ECM) breakdown (Spuijbroek *et al.*, 1992; Bruner *et al.*, 1997). Matrix metalloproteinases (MMPs) are essential in the remodelling of the ECM in normal development, growth and repair of tissues, and are implicated in cancer and in inflammatory and degenerative diseases.

MMPs, a large family of zinc-dependent, structurally related endoproteases can be distinguished in 5 subclasses according to structure and function, i.e. collagenases, gelatinases, stromelysins, membrane-type (MT-)MMPs and other MMPs. A survey of the known MMPs, their substrates and their tissue inhibitors (TIMPs) is presented in Table I. The possible role of MMPs and TIMPs in endometriosis has been discussed in various reports (Osteen *et al.*, 1996, 1999, 2002; Sillem *et al.*, 1998; Sharpe-Timms and Cox, 2002). Table 2 presents an overview of recent studies with regard to the relationship between MMP expression and endometriosis. The results of these studies are difficult to compare since different techniques are used, and only a selection of MMPs is studied in different case and control groups. Moreover, the results of some of these studies are conflicting. Although the expression of a number of MMPs is described, information on the function of these enzymes in the initial steps of the development of endometriosis is lacking.



The early phases in endometriotic lesion formation, when MMP activity is supposed to be particularly essential, are difficult to study in man *in vivo*. Recently, the functions of some MMPs in endometriosis have been evaluated in a murine model of experimentally induced endometriosis (Bruner *et al.*, 1997, 2002). These studies demonstrated that endometriosis-like lesion formation in this model can be prevented by inhibition of MMPs. However, no specific information with regard to MMP types was obtained, since the inhibitory actions affected a wide range of MMPs, and the relevance of these studies for the human situation is limited.

*Table 1. MMPs and their substrates.*

| MMP Subclass | MMP # | MMP name | Substrate |
|---|---|---|---|
| Collagenases | | | |
| | MMP-1 | Interstitial collagenase | Collagens I, II, III, VII, VIII, X; gelatin; aggrecan; L-selectin; IL-1β; proteoglycans; entactin; ovostatin; tenascin; TNF-α; MMP-2; MMP-9 |
| | MMP-8 | Neutrophil collagenase | Collagens I, II, III, V, VII, VIII, X; gelatin; aggrecan; fibronectin; entactin; tenascin |
| | MMP-13 | Collagenase-3 | Collagens I, II, III, IV, VII, IX, X, XIV; gelatin; plasminogen; aggrecan; perlecan; fibronectin; osteonectin; MMP-9 |
| | MMP-18 | Collagenase-4 | |
| Gelatinases | | | |
| | MMP-2 | Gelatinase A | Gelatin; elastin; fibronectin; collagen I, IV, V, VII, X, XI, XIV; fibronectin; TNF- α; aggrecan; osteonectin; MBP; laminin-1; MMP-1; MMP-9; MMP-13 |
| | MMP-9 | Gelatinase B | Collagens I, IV, V, VII, X, XI, XIV; gelatin; entactin; aggregan; elastin; fibronectin; osteonectin; plasminogen; MBP; IL-1 β; TNF- α |
| Stromelysins Stromelysins | | | |
| | MMP-3 | Stromelysin 1 | Collagens III, IV, V, IX, X, XI; fibrin/fibrinogen; tenascin; gelatin; aggrecan; perlecan; decorin; laminin; elastin; casein; osteonectin; ovostatin; entactin; plasminogen; MBP; IL-1β; proteoglycans; fibronectin; proteoglycans; MMP-1; MMP-2/TIMP-2; MMP-7; MMP-8; MMP-9; MMP-13 |
| | MMP-10 | Stromelysin 2 | Collagens III, IV, V, IX, X, XI; gelatin; casein; aggrecan; elastin; proteoglycans; fibronectin; laminin; fibrin/fibrinogen; entactin; tenascin; vitronectin; MMP-1; MMP-8 |
| | MMP-11 | Stromelysin 3 | Laminin; fibronectin; aggrecan |
| Membrane-type MMPs | | | |
| | MMP-14 | MT1-MMP | Collagens I, II, III; gelatin; casein; fibronectin; laminin; vitronectin; entactin; proteoglycans; aggrecan; MMP-2; MMP-13 |
| | MMP-15 | MT2-MMP | Fibronectin; entactin; laminin; perlecan; gelatin; vitronectin; collagen; aggrecan; MMP-2 |
| | MMP-16 | MT3-MMP | Collagen III; gelatin; casein; fibronectin; vitronectin; aggrecan; MMP-2 |
| | MMP-17 | MT4-MMP | Gelatin |
| | MMP-24 | MT5-MMP | Gelatin; fibronectin; vitronectin; collagen; aggrecan |
| | MMP-25 | MT6-MMP | Gelatin; collagen IV, fibrin; fibronectin; laminin-1; pro-gelatinase-A |

| MMP Subclass | MMP # | MMP name | Substrate |
|---|---|---|---|
| Others | | | |
| | MMP-7 | Matrilysin | Collagens III, IV, V, IX, X, XI; gelatin; aggrecan; decorin; fibronectin; laminin; entactin; elastin; casein; transferrin; plasminogen; MBP; β4-integrin; proteoglycans; fibrin/fibrinogen; tenascin; vitronectin; uPA; TNF- α; MMP-1; MMP-2; MMP-9; MMP-9/TIMP-1 |
| | MMP-12 | Metalloelastase | Collagen IV; gelatin; elastin; casein; fibronectin; vitronectin; laminin; entactin; MBP; fibrin/fibrinogen; plasminogen; proteoglycan |
| | MMP-19 | Unnamed | Gelatin; tenascin; fibronectin; collagen IV; laminin; entactin; fibrin/fibrinogen; aggrecan |
| | MMP-20 | Enamelysin | Amelogrenein; aggrecan; |
| | MMP-23 | Unnamed | Gelatin |
| | MMP-26 | Endometase | Gelatin I α; PI |

*Abbreviations*: IL: interleukin; MBP: myelin basic protein; MMP: matrix metalloproteinase; PI: protein inhibitor; TIMP: tissue inhibitor of MMP; TNF-α: tumour necrosis factor-α; uPA: urokinase-type plasminogen activator. *References*: McCawley and Matrisian, 2001; Whittaker and Ayscough, 2001.

The chicken chorioallantoic membrane (CAM) model has been used to study the invasive, metastatic and angiogenic potential of neoplastic cells (Scher *et al.*, 1976; Armstrong *et al.*, 1982), and can be used to differentiate between non-invasive and invasive epithelial cells (Schroyens *et al.*, 1989). Collagen type I and IV, laminin, and fibronectin are present in the ECM of the CAM (Giannopoulou *et al.*, 2001), which is similar to the ECM of the human peritoneum (Witz *et al.*, 2001). We have reported earlier that transplantation of human endometrium onto the CAM results in the formation of endometriosis-like lesions with endometrial glands and stroma of human origin (Maas *et al.*, 2001). The early phase of endometriosis-like lesion formation in this model is a rapid, proliferation-independent process, in which extensive tissue remodelling has been observed (Nap *et al.*, 2003). Consequently, we hypothesize that the expression of MMPs and TIMPs in human endometriosis is reflected in experimental endometriosis-like lesions in the CAM and that the CAM model is a helpful tool to evaluate functional involvement of MMPs in the development of endometriosis.



To test this hypothesis, the expression of MMPs -1, -2, -3, -7, -8, -9, -10, -11, -13, -14, -15, -16, -23, and of TIMP-1, -2 and -3 was evaluated in menstrual endometrium, in endometriosis-like lesions in the CAM and in human peritoneal and deep invasive endometriosis using semiquantitative immunohistochemistry. Subsequently, the MMP expression profile of experimentally induced endometriosis

in the CAM was compared to that of human endometriosis in order to see whether these profiles were similar. Finally, as expression of MMPs does not provide information about the activity of these enzymes, a functional study in the CAM was performed in which an MMP inhibitor was applied after transplantation of menstrual endometrium onto the CAMs. We selected a broad spectrum inhibitor which antagonizes the MMPs that were prominently present in the menstrual endometrium, the tissue that is thought to adhere and implant ectopically and to develop into endometriosis. The inhibiting effect on endometriosis-like lesion formation in the CAM was evaluated.

*Table 2. MMPs and endometriosis in literature*

| Author | MMPs TIMPs | technique | Material | Results | Phase of cycle |
|---|---|---|---|---|---|
| Kokorine *et al.*, 1997 | MMP-1 | ISH | Red and black lesions, OE, and RVE | MMP-1 present in red PE and OE, not in black PE and RV lesions | Various days |
| Wenzl *et al.*, 1998 | MMP-2 | IHC | Eut and ect from pat, eut from co | MMP-2 higher in ect than in eut in pat; no difference in eut between pat and co | Unknown |
| Gottschalk *et al.*, 2000 | MMP-1,2,3,9 TIMP-1,2 | IHC, ISH | Eut and ect from pat | MMP-1 higher, TIMP-1, -2 lower in ect than in eut | Various days |
| Chung *et al.*, 2001 | MMP-9 TIMP-3 | PCR | Eut and ect from pat, eut from co | Lower TIMP-3 in eut and ect from pat than in eut from co; higher MMP-9 and higher MMP-9/TIMP-3 ratio in ect than in eut from pat and co | Various days |
| Sillem *et al.*, 2001 | MMP-1,2,3,9 TIMP-1,2 | ELISA | Eut from pat and co; treatment with diethyl stilbestrol, TNF-α, IL-1, promegestone | MMP-1, -3 reduced by promegestone in pat and co; MMP-2 not influenced. MMP-3 upregulated by TNF-α in all samples, but by IL-1 in pat only. | Various days |
| Bruner-Tran *et al.*, 2002 | MMP-3,7 | ISH, WB | Eut and ect from pat and eut from co | MMP-3, MMP-7 similar in ect and eut (proliferative); MMP-3, MMP-7 absent in normal secretory eut but present in eut and ect from pat | Various days |
| Chung *et al.*, 2002 | MMP-2,14 TIMP-2 | RT-PCR, Z, WB | Eut and ect from pat; eut from co | MMP-2 and -14 higher, and TIMP-2 lower in eut from pat than from co | Unknown |
| Liu *et al.*, 2002 | MMP-9 | Z | Eut and ect from pat | MMP-9 higher in ect than in eut from patients, and higher in severe ect | Unknown |
| Mizumoto *et al.*, 2002 | MMP-1,2,3,7,9 TIMP-1 | IHC, WB, ELISA | OE fluid from pat and eut from co | In OE: MMP-1, -2, -7, -9, TIMP-1 present in menstrual phase; MMP-3 in macrophages in all phases of cycle | Various days |

| Author | MMPs TIMPs | technique | Material | Results | Phase of cycle |
|---|---|---|---|---|---|
| Nezhat *et al.*, 2002 | MMP-9 | IHC | Ect and OE | MMP-9 more expressed in ect than in OE | Unknown |
| Ria *et al.*, 2002 | MMP-2,9 | ISH | Stage III and IV OE from pat and eut from co | MMP-2, -9 higher in OE stage IV than stage III; higher in OE than in eut from co | Pat: unknown; Co: midsecretory |
| Szamatowicz *et al.*, 2002 | MMP-9 MP-1 | ELISA | PF from pat vs. PF from co | Active MMP-9 the same in PF of pat and co; TIMP-1 lower in PF of pat than of co | Days 8-12 |
| Ueda *et al.*, 2002 | MMP-2,9,14 | IHC, RT-PCR | Ect from pat and eut from co | MMP-2, -9, -14 higher in ect from pat than in eut from co | Unknown |
| Gilabert-Estelles *et al.*, 2003 | MMP-3 TIMP-1 | ELISA | Eut, and PF from pat and co, OE from pat | MMP-3 higher, TIMP-1 equal in eut from pat compared to co; MMP-3 higher, TIMP-1 lower in eut than in OE from pat; no differences in PF | Various days |
| Wolber *et al.*, 2003 | MMP-1,2 | PCR | Eut from co cultured on CAM | MMP-1 upregulated; MMP-2 unaltered after culturing eut on CAM | Various days |

*Abbreviations*
IHC: Immunohistochemistry, ISH: In Situ Hybridisation, (RT-)PCR: (Reverse Transcriptase-) Polymerase Chain Reaction, WB: Western Blot, Z: Zymography, Co: Control women without endometriosis, Pat: Patients, Ect: Ectopic endometrium or endometriosis, Eut: Eutopic endometrium, OE: Ovarian Endometriosis, PE: Peritoneal Endometriosis, RV: Rectovaginal Endometriosis, PF: Peritoneal Fluid.

## Materials and methods



### Tissue

*Human endometrial tissue*

Endometrium was collected by biopsy from 10 women with normal ovulatory cycles, undergoing laparoscopy for benign conditions. No visible endometriosis was present. An Endobiops endometrium sampling device (Gynotec, Malden, The Netherlands) was used. Endometrium was collected during the menstrual phase of the cycle (1 to 3 days after the onset of bleeding). The use of human endometrium was approved by the Institutional Ethical Review Committee of the University Hospital Maastricht. All women participating in the study gave their written informed consent. Immediately after collection, blood clots were removed and the endometrium was carefully minced in fragments of 1-2 $mm^3$ and kept in serum-free DMEM/HAM's F12 culture medium supplemented with 2 mmol/l L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (all from Gibco Life Technologies, Breda, The Netherlands). Part of the biopsied menstrual

endometrium was used for transplantation onto the CAM. Of each endometrium biopsy, tissue was embedded in paraffin, sectioned, Haematoxylin & Eosin (H&E) staining was performed for histological evaluation and to assess tissue integrity, and immunohistochemical analysis for MMP expression was performed.

*Archival human endometriotic tissue*

Paraffin blocks of red peritoneal lesions (n=5) and of endometriosis located in the rectovaginal space (n=5) collected from patients undergoing surgery in order to remove their endometriosis, were obtained from the archives of the Department of Pathology of the University Hospital Maastricht. Sections were prepared and stained with H&E to confirm the presence of endometriotic lesions.

**Chorioallantoic membrane model**

Fertilised eggs of Leghorn chickens were incubated and prepared as described previously (Nap *et al.*, 2003). Fragments of biopsied menstrual endometrial tissue were transplanted onto the CAM. After 72 hours of incubation the area of the CAM containing the endometrial fragment was excised, fixed in 3.7% buffered formaldehyde and embedded in paraffin. The entire specimen was sectioned (4 μm, 150-200 sections). Every fifth section was stained with H&E for histological evaluation to identify the lesion.

**Immunohistochemistry**



Immunohistochemistry was performed on paraffin sections from biopsied menstrual endometrium, from endometriosis-like lesions in CAMs and from archival peritoneal and deep invasive endometriotic tissue.

Expression of MMPs was evaluated by immunohistochemistry using the commercially available antibodies against human MMPs which were suitable to stain paraffin-embedded tissue (Oncogene, La Jolla, CA, USA) (Appendix 1). These included MMPs -1, -2, -3, -7, -8, -9, -10, -11, -13, -14, -15, -16, -23, and TIMPs -1, -2 and -3. All antibodies were monoclonal mouse antibodies, except anti-MMP-23, which was a polyclonal rabbit antibody. The antibody dilutions were optimized for application on routinely-fixed, paraffin-embedded tissue sections using breast cancer, colon cancer and placental tissue as positive controls. For each MMP, all tissues were included in 1 staining procedure in order to keep variability to a minimum. In short, paraffin sections were deparaffinised in xylene for 2 x 3 minutes and rehydrated in alcohol series. Endogenous peroxidase activity was blocked by incubation with 0.25% hydrogen peroxide in methanol for 20 minutes. Sections were rinsed 3 times in phosphate-buffered saline (PBS). For incubation with primary antibodies against MMPs -1, -3, -7, and -23 antigen retrieval in Tris EDTA buffer (20

minutes at 95°C) was required. After rinsing again in PBS, sections were incubated overnight at 4°C (except for the antibodies against MMPs-10 and -16 which were incubated for 2 hours at room temperature) with the appropriately diluted primary antibodies. After 3 rinses in PBS, sections were exposed to Envision Chemmate (DAKO, Glostrup, Denmark) for 30 minutes. After rinsing again in PBS, antibody binding was visualised with 3'-3-diaminobenzidin (DAB). Sections were washed and counterstained with haematoxylin, washed again, dehydrated and mounted for light microscopic evaluation. Sections in which the primary antibody was left out of the immunostaining procedure served as negative controls.

### Evaluation of immunostaining

Immunostaining was assessed semi-quantitatively using a staining index. The staining was evaluated by 3 independent observers at a magnification of 400 x. Menstrual endometrial specimens and the CAM lesions were evaluated entirely. In the tissue containing endometriosis, a representative lesion was selected and evaluated by all observers.

The semiquantitative staining index was calculated according to the following equation: Proportion of stained cells x Staining intensity. The proportion of stained cells was expressed as 0, 1, 2, or 3 (0%, <10%, 10-50% or >50% of cells, respectively), and intensity of staining as 1, 2, or 3 (weak, moderate, or strong, respectively). The minimum score was 0 and the maximum score was 9. The threshold for positive staining was set at a staining index of 1. The average of the scores of the 3 observers was calculated and taken as the staining index for that particular section. Intra- and interobserver variability was 9%. Since all MMPs except MMP-7 are predominantly present in the stroma (Hulboy *et al.*, 1997), staining indices were calculated in the stromal compartment for all MMPs except for MMP-7. MMP-7 is known to be expressed in the epithelium (Hulboy *et al.*, 1997), in which tissue the staining index for MMP-7 was calculated.



### Inhibition of MMP activity

The effect of the inhibition of MMP activity on endometriosis-like lesion formation was studied by the administration of an MMP inhibitor that inhibits MMP -1, -2, -3, -7, and -13 (MMP inhibitor III, Catalog number 444264; Calbiochem, La Jolla, CA, USA), to the menstrual endometrial fragments after transplantation onto the CAM. MMP inhibitor III was dissolved in DMSO and diluted 1:1000 in normal saline (NaCl) to reach a final concentration of 1 μM (stock). Prior to the first application of the MMP inhibitor, endometrial fragments were allowed to attach to the CAMs for 1 to 2 hours in order to avoid mechanical removal of the fragments by flushing during the administration of the MMP inhibitor. Vehicle (diluted DMSO) was applied to 10

CAMs and did not change or damage the CAMs. The MMP inhibitor was administered 2 times on days 0, 1 and 2 (65 µl of the 1 µM stock). After incubation for 72 hours, CAMs were fixed, embedded in paraffin, sectioned and stained as described above. Endometriosis-like lesion formation was assessed in 28 CAMs treated with the MMP inhibitor and 86 control CAMs treated with vehicle.

### Statistical analysis

Minimum, maximum, median and range of all staining intensities were calculated. Differences between medians of staining indices were calculated using the Kruskal Wallis test for more than 2 independent variables. The number of endometriosis-like lesions developed in CAMs after treatment with an MMP inhibitor and the control CAMs treated with vehicle were compared using $\chi^2$ tests. P-values $<0.05$ were considered statistically significant.

## Results

### MMP and TIMP expression in menstrual endometrium, experimental endometriosis in the chorioallantoic membrane and in human peritoneal and deep invasive endometriosis

The staining index (SI) for MMP and TIMP expression was used to compare the MMP expression between experimental endometriosis in the CAM and human endometriosis. Differences in SI were found between endometriosis-like lesions in the CAM and human endometriosis in 3 out of 16 MMPs and TIMPs. Expression of MMP-1 ($p<0.05$), -16 ($p<0.05$) and TIMP-3 ($p<0.01$) was significantly lower in human endometriosis compared to experimental endometriosis in the CAM as shown in Figure 1a. No differences were found between the SIs of menstrual endometrium and the endometriosis-like lesions in the CAM.



A majority of the studied MMPs and TIMPs was expressed in the examined tissues, except MMP-8 which was absent in peritoneal endometriosis, and MMP-14 which was absent in all tissues. Representative examples of MMP staining are presented in Figure 2.

### Effect of inhibition of MMP activity on endometriosis-like lesion formation in the chorioallantoic membrane

MMP inhibitor III was selected to inhibit the activity of MMPs -1, -2, -3, -7, and -13, in order to evaluate its effect on the formation of endometriosis-like lesions in the CAM. This choice was based on the observation that MMPs -1, -2, -3, -7, -11, -13, -15, and -23 were the most abundantly expressed MMPs in menstrual endometrium

(Figure 1b). For MMPs -11, -15 and -23 no inhibitors were available. After 72 hours, endometriosis-like lesions were present in 32% of CAMs to which the MMP inhibitor was administered, whereas endometriosis-like lesions were observed in 76% of control CAMs ($p<0.05$).

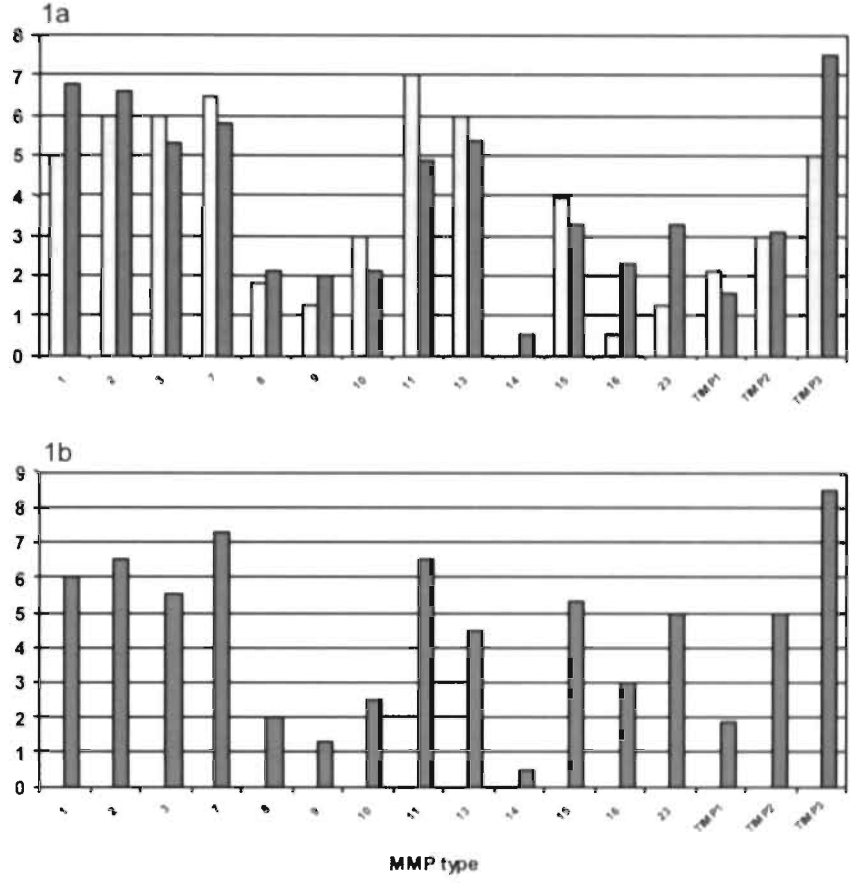


*Figure 1* *Staining indices of human endometriosis (light grey bars) and endometriosis-like lesions in the CAM (dark grey bars) (A), and of menstrual endometrium (B). Staining indices were determined exclusively in stromal cells for all MMPs and TIMPs except for MMP-7, in which staining indices were determined for epithelial cells.*

## Discussion

In this study we demonstrate for the first time the expression of the collagenases MMP-8 and -13, the stromelysin MMP-10, the membrane type-MMPs MMP-15 and -16, and MMP-23 in human endometrium and in endometriosis. Inhibition of MMP activity impairs the development of early endometriotic lesions in the CAM model.

In order to investigate whether the CAM was suitable to study expression and function of MMPs in endometriosis, we initially compared the MMP expression profile of peritoneal and deep invasive endometriosis with that of endometriosis-like lesions in CAMs. The expression profiles turned out to be very similar. In menstrual endometrial tissue, MMPs -1, -2, -3, -7, -11, -13, -15 and -23 showed the relatively highest expression levels, indicated by the staining index.

Inhibition of the majority of these MMPs led to significant inhibition of endometriosis-like lesion formation in CAMs. This indicates that MMPs are involved in endometriosis-like lesion development and that the CAM model is a useful tool to evaluate the involvement of MMPs in the initial steps of lesion formation of endometrial tissue. Lesion formation in the CAM was not completely prevented by the inhibitors used. This suggests that other MMPs or other mechanisms contribute to successful implantation of endometrium. Alternatively, it is possible that the inhibitor did not completely abolish all MMP activity.

Until recently MMPs were thought to function primarily as regulators of ECM composition and structure and to facilitate cell migration and invasion by removing barriers such as collagen. It is now becoming evident that MMPs cleave a variety of substrates that are not ECM components (Stamenkovic, 2003). Therefore, MMPs may be implicated in the regulation of (latent) growth factors, cytokines, angiogenesis, invasion, tissue organization and cell survival (Stamenkovic, 2003). The high expression of various MMPs in the endometriotic lesions may indicate the presence of an alternative self-sustaining mechanism of the endometriotic tissue, next to the local production of estrogens (Gurates and Bulun, 2003).

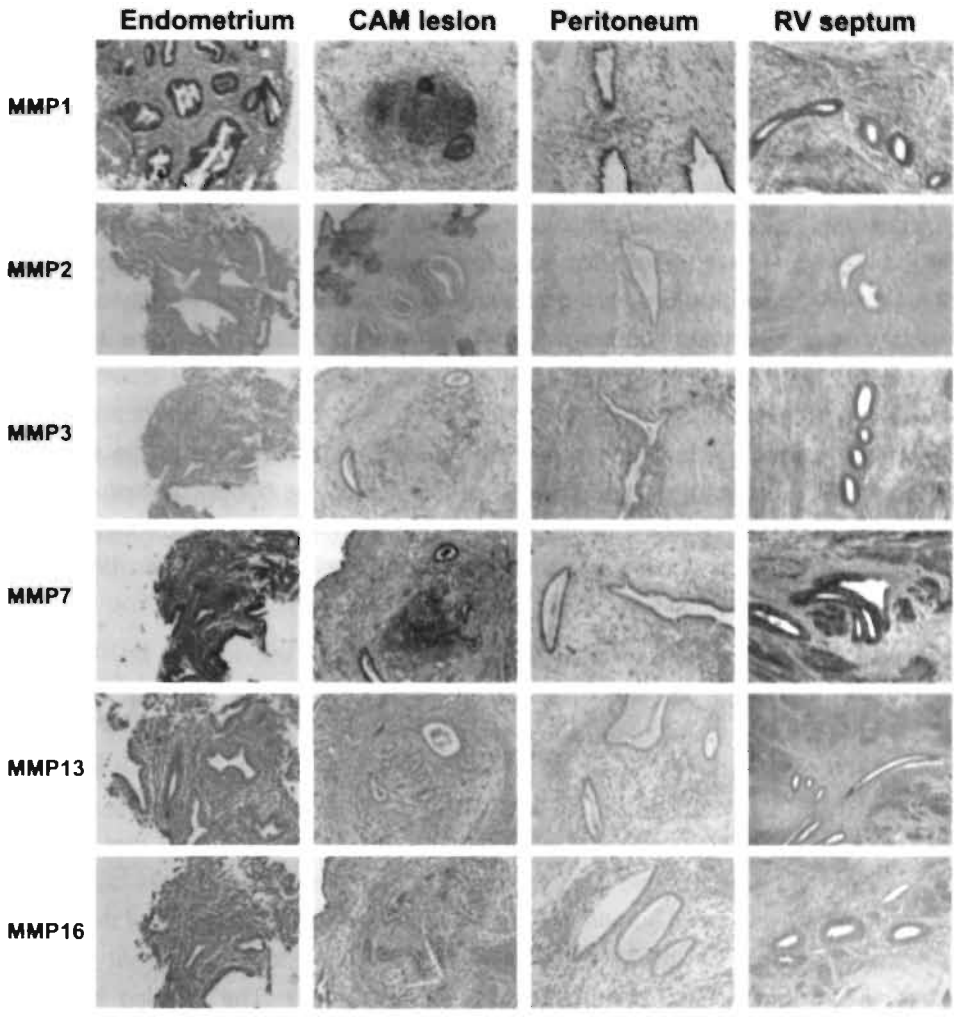


*Figure 2. Representative examples of immunohistochemical staining of MMPs in menstrual endometrium, CAM lesions, peritoneal endometriosis and deep invasive endometriosis.*

Transplantation of menstrual endometrium onto the CAM did not lead to upregulation of MMP expression in the endometriosis-like lesions that developed in CAMs. Apparently, the MMP expression in the endometrial tissue itself is an important determinant in the process of lesion formation, and not the local environment. This is in agreement with the study of Osteen and co-workers who stated that misregulation of the paracrine mediators of MMPs in the eutopic endometrium may be a central discriminating factor for the propensity of endometrium to implant ectopically, as a consequence of -endogenous or exogenous- steroid exposure, environmental toxin exposure, immunological

disturbances or genetic predisposition (Osteen *et al.*, 2002). This is also supported by the observation that members of all MMP subclasses as well as all TIMPs were present and that there were no dramatic differences in the expression of MMPs in eutopic and ectopic endometrium, and in experimentally induced endometriosis-like lesions in CAMs.

To date, the immunohistochemical study of a large series of MMPs was hampered by the limited availability of antibodies applicable for staining paraffin-embedded tissue. In this study we have used all antibodies that were commercially available. Special care was taken to quantify the expression and to minimize bias and variability. Since the active forms of MMPs and TIMPs are usually located in the stromal compartments of tissues, we focussed on the presence of MMPs and TIMPs in the stroma. However, substantial MMP staining was observed in the epithelial cells as well. The expression of MMPs in epithelial cells is not uncommon and has been described earlier in endometrial epithelial cells (Henriet *et al.*, 2002), human keratinocytes (Pilcher *et al.*, 1997) and mammary epithelium (Witty *et al.*, 1995). The significance of these epithelial MMPs is not yet known.

The presence of the collagenases MMP-8 and -13, the stromelysin MMP-10, the membrane type-MMP-15 and -16, and of MMP-23 was not reported before in endometrium and in endometriosis, but has been described in a variety of other tissues and processes (Balbin *et al.*, 1999; Pendas *et al.*, 1999; Pilcher *et al.*, 1999; Rechtman *et al.*, 1999; Sorsa *et al.*, 1999; Velasco *et al.*, 1999; Hernandez-Barrantes *et al.*, 2002; Leeman *et al.*, 2002). The present study shows that MMP-13 and -23 are highly expressed in menstrual endometrium, endometriosis-like lesions in the CAM as well as in human endometriosis. As MMP-13 was one of the MMPs effectively inhibited by the MMP inhibitor, it is tempting to suggest that MMP-13 plays a role in the early pathogenesis of endometriosis. The staining index for MMP-9 and MMP-14 was low, in menstrual endometrium as well as in human and experimental endometriosis. Ueda and co-workers did not find high MMP-9 and -14 expression in endometrium from women without endometriosis. However, in contrast to our findings, they detected high expression levels in ectopic endometrium from endometriosis patients (Ueda *et al.*, 2002). The expression of MMPs -1, -2, -3, -7, -11 and TIMPs -1, -2, and -3 is in accordance with previous reports (Osteen *et al.*, 1996, 1999, 2002; Sillem *et al.*, 1998; Sharpe-Timms and Cox, 2002; Table 2). Based on the present study and literature reports, we conclude that a combined action of MMPs of all subclasses may be responsible for the development of endometriosis.

In summary, we have assessed the expression of a broad range of MMPs and TIMPs in human endometrium and endometriosis, and in experimentally induced

endometriosis-like lesions. The expression patterns of MMPs in these tissues appear to be similar. Also, it has been shown that inhibition of most of the abundantly expressed MMPs in menstrual endometrial tissue effectively reduces endometriosis-like lesion formation in the CAMs. Therefore, the CAM model was found suitable to study MMP expression and function in lesion development in endometriosis. Our results indicate functional involvement of MMPs -1, -2, -3, -7 and -13 in this process, however, the involvement of other MMPs or mechanisms cannot be excluded.

## Acknowledgements

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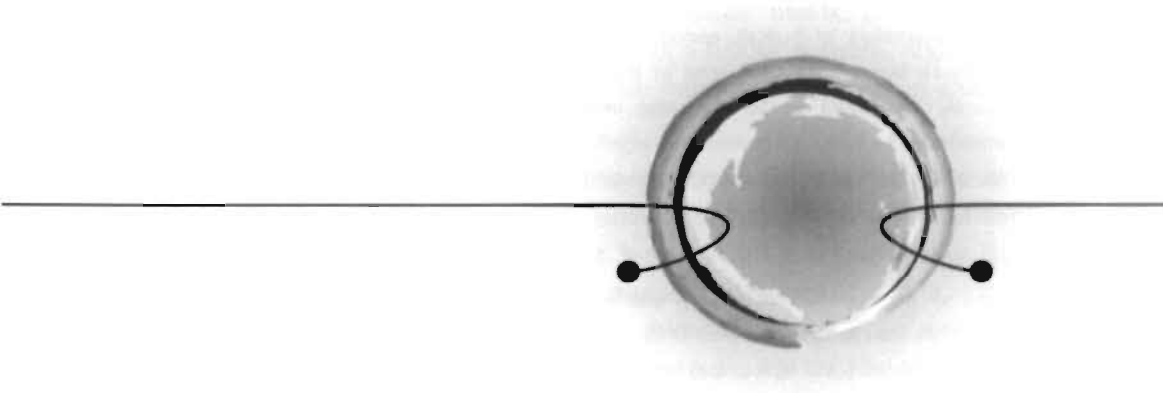
# Chapter 4

## Anti-angiogenic agents prevent the development of endometriosis in the chicken chorioallantoic membrane



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Arjan W. Griffioen, Johannes L.H. Evers,
and Gerard A.J. Dunselman

Submitted

# Abstract

**Objective**

To assess whether inhibition of angiogenesis by anti-angiogenic therapy inhibits the development of endometriosis, assuming that angiogenesis is essential in its pathogenesis.

**Design**

Prospective study to determine the effects of the angiostatic compounds anti-hVEGF, TNP-470, endostatin and anginex on the vascularisation and on endometriosis-like lesion formation in the chicken chorioallantoic membrane (CAM) model.

**Setting**

Tertiary care medical center and university research laboratory.

Animals: day 10 chicken embryo's onto which human endometrium was transplanted. Interventions: Regular administration of angiostatic compounds to the CAMs for 72 hours.

**Main outcome measures**

Vascular density indices, endometriosis-like lesion formation and morphology of endometriosis-like lesions.

**Results**

After transplantation of human endometrium onto the CAM, vessel densities increased and endometriosis-like lesions developed in the CAM. Administration of angiostatic agents caused *significantly decreased vessel densities* in the CAM after transplantation of endometrium. Endometriosis-like lesion formation was significantly impaired after treatment with anti-angiogenic agents, and significantly more necrosis was present in endometriosis-like lesions treated with inhibitors of angiogenesis.

**Conclusion**

Inhibition of angiogenesis effectively interferes with the vascularisation and the formation of endometriosis-like lesions in the CAM, demonstrating that endometriosis is dependent on angiogenesis. Angiostatic agents may be promising as future therapeutic options for endometriosis.

## Introduction

Endometriosis is one of the most frequently encountered gynaecological problems (Gazvani and Templeton, 2002). It is defined as the presence of endometrium outside the uterine cavity, and supposedly is the result of the implantation of retrogradely shed endometrium during menstruation (Sampson, 1927). Similar to tumour implants that are not capable of growing beyond a volume of 3 $mm^3$ unless they develop a new blood supply, ectopically implanted endometrium may trigger an angiogenic switch, activating angiogenesis within and around the tissue (Abulafia and Sherer, 1999). Circumstantial evidence in literature suggests that angiogenesis is a prerequisite for the development of endometriosis. Endometrium has angiogenic potential (Maas *et al.*, 1999), increased angiogenesis is found around peritoneal implants (Nisolle *et al.*, 1993), and increased endothelial cell proliferation has been observed in eutopic endometrium of endometriosis patients (Wingfield *et al.*, 1995). Moreover, peritoneal fluid has an increased angiogenic activity in women with endometriosis compared to women without the disease (Oosterlynck *et al.*, 1993). Angiogenesis is the formation of new blood vessels from pre-existing capillaries. It involves the proteolytic degradation of the extracellular matrix, proliferation and migration of endothelial cells, and the formation of a tubular network. Angiogenesis is essential in wound healing and in the female reproductive system in processes including the menstrual cycle and pregnancy.

Since angiogenesis may be essential in the pathogenesis of endometriosis, we hypothesize that inhibition of angiogenesis leads to inhibition of the development of endometriotic lesions. This hypothesis was tested in the chicken chorioallantoic membrane (CAM) model, which has been proven to be a suitable model to study the angiogenic properties of human endometrium (Maas *et al.*, 1999) as well as the development of endometriosis-like lesions (Maas *et al.*, 2001a; Nap *et al.*, 2003). We evaluated the vascularisation and endometriosis-like lesion formation after transplantation of human endometrium together with angiostatic agents on the CAM. Since vascular endothelial growth factor (VEGF) was demonstrated to be the predominant angiogenic factor in human endometrium (Charnock-Jones *et al.*, 1993), we assessed the function of this growth factor by blocking it with a humanized VEGF antibody (anti-hVEGF). In addition to anti-hVEGF, we used 3 other angiogenesis inhibitors, the fungus derived antibiotic TNP-470 (Ingber *et al.*, 1990), and the specific angiogenesis inhibitors endostatin (O'Reilly *et al.*, 1997) and anginex (Mayo *et al.*, 1996; Griffioen *et al.*, 2001; Van der Schaft *et al.*, 2002).

## Materials and Methods

### Endometrial tissue

Proliferative endometrium (days 5 to 11 of the menstrual cycle) was collected in 15 women by transvaginal biopsy using a sampling device (Gynotec, Malden, The Netherlands). Women were 25 to 42 years of age, they had regular menstrual cycles, and none had symptomatic endometriosis. Gynaecological pathology was found in none of the endometrial biopsies. The use of human endometrium was approved by the Institutional Ethical Review Committee of the University Hospital Maastricht. All women gave written informed consent. Immediately after collection, blood clots were removed, the endometrium was minced in small fragments of 1-2 $mm^3$ and kept in serum-free DMEM/HAM's F12 culture medium. From each endometrial biopsy, tissue was embedded in paraffin and sectioned. Haematoxylin and Eosin (H&E) staining was performed for histologic evaluation. The phase of the menstrual cycle was histologically confirmed by a pathologist.

### Reagents

HuMV833, a humanized VEGF antibody (anti-hVEGF) was provided by Protein Design Labs (Fremont, USA). TNP-470 (AGM-1470) was purchased from Takeda Chemical Industries (Osaka, Japan). Endostatin was provided by Entremed Inc. (Rockville, MD., USA). Anginex was synthesized (Griffioen *et al.*, 2001) and provided by K.H. Mayo (Minneapolis, MN).



### Chorioallantoic membrane model

Fertilised eggs of Lohman-selected White Leghorns were prepared as described previously (Nap *et al.*, 2003). On day 7 of incubation, a 10 mm silicon ring was placed on the CAM to allow drug administration of 65 µl quantities within the ring. On day 10 of incubation, an endometrium fragment of 1-2 $mm^3$ was transplanted within the ring. Two hours after transplantation, anti-hVEGF (3 mg/kg/day, n=22), TNP-470 (20 mg/kg, once every 2 days, n=22), endostatin (2 mg/kg/day, n=10), anginex (8 mg/kg/day, n=24) or vehicle (65 µl of saline (NaCl)/day, n=10) were administered. For each reagent, the same treatment regimen was applied to 10 CAMs without endometrial fragment to study the effect of the reagent on CAM morphology and on its vascularisation. For the calculation of the right dose, the average weight of a chicken embryo after 10 days of incubation was taken.

Seventy two hours after transplantation of the endometrial fragment onto the CAM, color micrographs of the area within the ring were made as described previously (Maas *et al.*, 2001b). Subsequently CAMs were fixed by injecting 1 ml of 3.7%

buffered formaldehyde on top of and 1 ml under the CAM, and the area of the CAM containing the endometrial fragment was excised and embedded in paraffin. Paraffin sections (4 μm) were cut from the entire specimen (150-200 sections) and sections were H&E stained for histological evaluation.

### Analysis of angiogenesis and endometriosis-like lesion development in the CAM

The vascular density index (VDI) was calculated by superimposing a circular grid containing 5 concentric circles on the image of the CAM and counting the number of intersections of the rings and the blood vessels (Maas *et al.*, 2001b). Angiogenesis present in CAMs without endometrial fragment was defined as developmental angiogenesis. Angiogenesis occurring after transplantation of endometrium onto the CAM was defined as endometrium-induced angiogenesis.

Endometriosis-like lesions, studied microscopically on H&E stained sections, were defined as the presence of human endometrial glands and endometrial stroma in the CAM mesenchyme (Maas *et al.*, 2001a; Nap *et al.*, 2003). Percentage of necrosis in endometriosis-like lesions was quantified using a microscope coupled to a computerized morphometry system (Quantimet 570, Leica, the Netherlands). Sections were evaluated twice under identical circumstances, and the average of the evaluations was taken.

### Statistical analysis

Descriptive statistics (median, range and percentiles) were calculated for each experimental group, and compared using the non-parametric Mann-Whitney-U test. Dichotomous variables were compared using $\chi^2$ tests. Correlations were calculated using Spearman correlation tests. P-values $<0.05$ were considered statistically significant. Vascular density indices were counted blindly, twice under identical circumstances, by 2 observers. The average of the counts was taken. Intra-observer and inter-observer variabilities were between 5 and 10%.



## Results

### Human endometrium is angiogenic

Transplantation of human endometrium onto the CAM led to a strong angiogenic response in the chicken tissue, both at the level of vessel number as well as at the level of vessel architecture (Figure 1a and 1b), and to the formation of endometriosis-like lesions (Figure 2a), as we have demonstrated before (Maas *et al.*, 1999; Nap *et al.*, 2003).

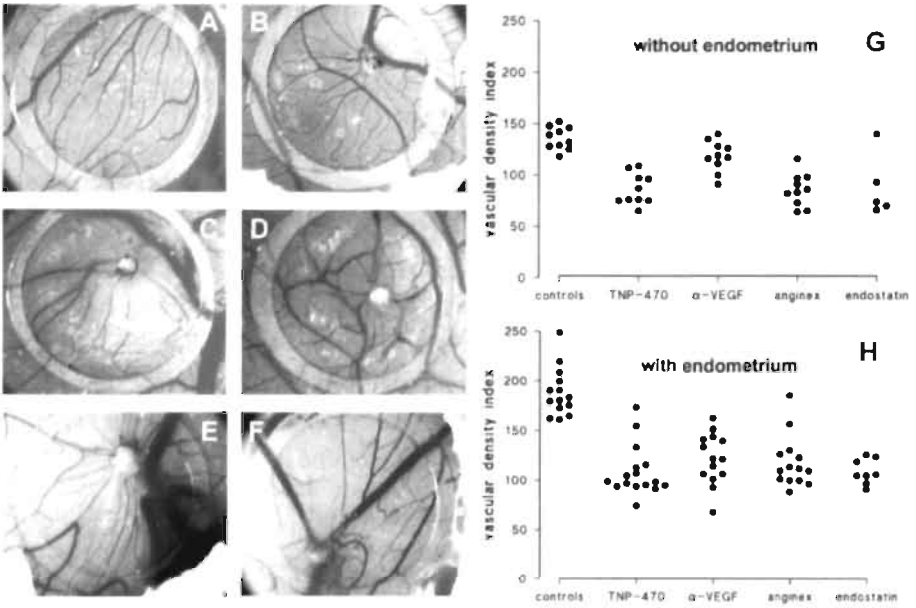


*Figure 1* *Vessel density and vessel architecture in CAMs after administration of angiostatic agents. In ovo photograph of a CAM on day 13 of development. 1A: Control CAM without transplanted human endometrial fragment. 1B: Endometrium-induced angiogenesis (with transplanted human endometrial fragment). Note the significant increase in vascular density index in the CAM transplanted with an endometrial fragment. The inhibitory effects on the vascularisation by anti-hVEGF (1C), TNP-470 (1D), endostatin (1E), and anginex (1F). The number of vessels is significantly decreased, and vessel architecture is disturbed after administration of angiostatic agents. Panel 1G shows the inhibitory effect of the angiostatic agents on the vascular density index (VDI) in CAMs without transplanted endometrial fragment (developmental angiogenesis). Developmental angiogenesis is decreased after administration of TNP-470, endostatin and anginex, but not after administration of anti-hVEGF. In panel H, VDI of endometrium-induced angiogenesis is shown. All angiostatic agents cause a significant decrease of the endometrium induced angiogenesis.*



Endometrium-induced angiogenesis in the CAM is suppressed by angiostatic agents. In order to study the significance of the angiogenic potential of endometrium in the formation of endometriosis-like lesions, several angiogenesis inhibitors were selected for intervention treatments in the CAM model. Since VEGF-A has been demonstrated to be the predominant angiogenic factor (Charnock-Jones *et al.*, 1993), we studied the function of this growth factor by blocking it with a humanized VEGF-A antibody (anti-hVEGF). No effect of anti-hVEGF was observed in the vascularisation of CAMs without an endometrial fragment (Figure 1g). The efficient angiogenic response to the presence of transplanted human endometrium (Figure 1b) was significantly inhibited by treatment with this antibody. This was measured at the level of the vascular density index (VDI) (Figure 1c and 1h). In addition to anti-hVEGF, CAMs were treated with 3 other angiogenesis inhibitors, i.e. the fungus

derived antibiotic TNP-470 (Figure 1d), and the specific angiogenesis inhibitors endostatin (Figure 1e) and anginex (Figure 1f). All 3 compounds significantly inhibited the developmental angiogenesis in the CAM by 45% ($p<0.05$), 51% ($p<0.03$), and 48% ($p<0.01$), respectively (Figure 1g).

The endometrium induced angiogenesis was inhibited as well by these angiogenesis inhibitors, by 43% ($p<0.05$), 43% ($p<0.03$), and 38% ($p<0.01$), respectively (Figure 1h).

**Endometriosis-like lesion formation is prevented by angiostatic agents in the CAM model**

Transplantation of a human endometrial fragment onto the CAM resulted in the formation of endometriosis-like lesions in a majority of CAMs (Figure 2). In vehicle treated control CAMs, this was observed in 20 out of 24 CAMs (83%).

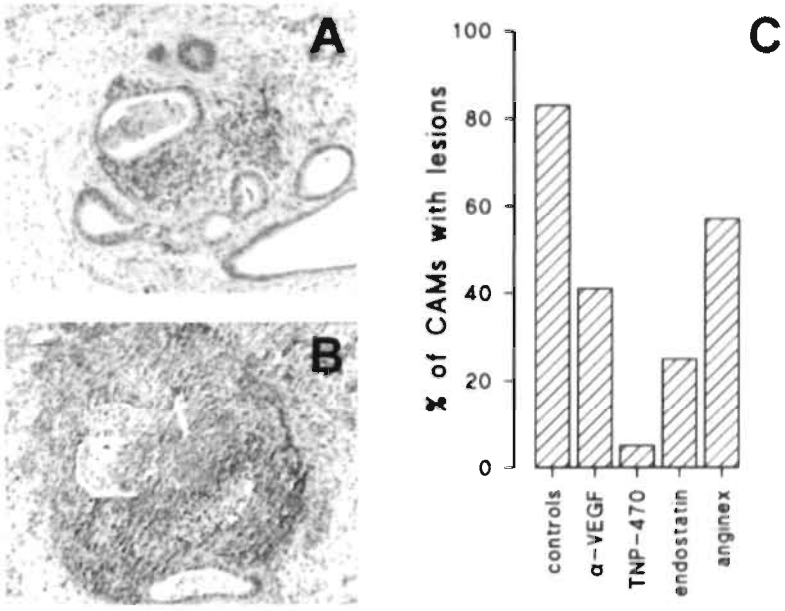


*Figure 2* *Formation and morphology of endometriosis-like lesions in CAMs after administration of angiostatic agents.*
*Panel A shows the morphology of an endometriosis-like lesion in a control CAM. Normally developed human endometrial stroma and glands are present, and no necrosis is observed (H&E staining). In panel 2B an endometriosis-like lesion after treatment with endostatin is shown. Normal morphology is disturbed. Note the massive amount of necrosis (H&E staining). 2C. After administration of angiostatic agents the formation of endometriosis-like lesions in CAMs is significantly inhibited.*

Administration of the angiostatic agents significantly reduced endometriosis-like lesion formation. Anti-hVEGF reduced lesion formation to 41% (9/22, $p<0.005$). In

CAMs treated with TNP-470 only 1 endometriosis-like lesion was observed in 22 CAMs (5%, $p<0.0001$). In a majority of CAMs treated with TNP-470, a disturbed morphology of the surface of the CAMs was observed. In CAMs treated with endostatin, endometriosis-like lesion formation was reduced to 25% (2/8, $p<0.001$), and anginex treatment resulted in 13 out of 23 CAMs containing endometriosis-like lesions (57%, $p<0.05$). A significantly higher number of vessels was observed in CAMs in which an endometriosis-like lesion came to development, as compared to CAMs without a lesion ($p<0.05$), indicating the dependence of endometriosis-like lesion formation on development of new vessels.

All endometriosis-like lesions were histologically evaluated. Unlike endometriosis-like lesions in control CAMs, a significant part of endometriosis-like lesions in the CAMs treated with angiostatic agents showed necrotic morphology (Figure 2b). Treatment with anti-hVEGF, endostatin and anginex resulted in a necrotic area of 9.4% ($p<0.05$), 32% ($p<0.01$), and 16% ($p<0.01$) of the endometriosis-like lesion respectively, compared to 4% of the endometriosis-like lesion in control CAMs. The single lesion that was found in the 22 TNP-470 treated CAMs consisted of a single gland surrounded by a small covering layer of stroma, and 7% of the lesion had a necrotic morphology. A strong negative correlation was found between the VDI and the percentage of necrosis ($R= -0.63$, $p<0.0001$).

## Discussion



In this study we demonstrate that anti-angiogenic agents are able to inhibit the vascularisation of endometrial implants and thus diminish the formation of endometriosis-like lesions in the chorioallantoic membrane (CAM).

Developmental angiogenesis in the growing CAM was significantly inhibited by TNP-470, endostatin and anginex, whereas treatment with anti-hVEGF did not affect this embryological process. Because the anti-human VEGF antibody would only block human VEGF, no effects were expected in the CAM without human endometria; transplants. Indeed, no significant response in the CAM was observed. The efficient angiogenic response by transplanted human endometrium on the CAMs measured at the level of the VDI was strongly and significantly inhibited by treatment with anti-hVEGF and the other angiostatic agents, indicating that all applied agents effectively inhibited the endometrium-induced angiogenesis.

The use of angiogenesis inhibitors in the CAM led to a significantly impaired development of endometriosis-like lesions, which was considered to be caused by the inhibition of angiogenesis. An association between the VDI in CAMs and the

number of endometriosis-like lesions was observed. CAMs without an endometriosis-like lesion showed a significantly lower VDI than CAMs with a lesion. In addition, endometriosis-like lesions in CAMs treated with anti-hVEGF, endostatin or anginex contained significantly more necrosis as compared to control CAMs. The negative correlation between the VDI in CAMs and the percentage of necrosis in endometriosis-like lesions supports the idea that a disturbed vascularisation as a consequence of the presence of the inhibitors is responsible for the necrosis in the CAMs.

In this study, angiostatic agents which have not been used before in the CAM model for endometriosis have been tested on their inhibitory effects on angiogenesis and endometriosis-like lesion formation. Vascular endothelial growth factor (VEGF) is a major player in angiogenesis based on its ability to induce vasodilatation and its endothelial cell permeability increasing effect (Ziche *et al.*, 1997). In endometriosis, VEGF plays a pivotal role as well. VEGF-A content was significantly higher in the eutopic glandular epithelium of endometriosis patients during the late secretory phase as compared to that of women without endometriosis (Donnez *et al.*, 1998). Moreover, in menstrual effluent as well as in peritoneal endometriotic lesions VEGF-A was expressed (McLaren, 2000; Smith, 2001). In an animal study, it was demonstrated that inhibition of VEGF prevents endometriosis-like lesion formation in nude mice (Hull *et al.*, 2003). Recently, a humanized anti-VEGF antibody, Avastin, was administered for the first time in man in the treatment of colorectal carcinoma, with satisfactory results (McCarthy, 2003). This approach of neutralizing VEGF provided the first proof-of-concept that anti-angiogenesis is applicable in man. The major role of VEGF in endometriosis may also predict success of Avastin in the treatment of endometriosis. In our study, anti-hVEGF selectively inhibited endometrium-induced angiogenesis and effectively inhibited endometriosis-like lesion formation.



TNP-470 (AGM-1470) is a representative of the first generation anti-angiogenic agents. It is a fumagillin analogue and a naturally secreted antibiotic of Aspergillus Fumigatus Fresenius which inhibits endothelial cell proliferation *in vitro*, tumour induced angiogenesis *in vivo*, and tumour growth in mice (Ingber *et al.*, 1990; Yamaoka *et al.*, 1993; Bergers *et al.*, 1999). TNP-470 is one of the most potent inhibitors of angiogenesis and has been tested in clinical trials for treating a variety of malignancies. However, serious dose-related side effects limit the potential of TNP-470 (Bergers *et al.*, 1999). In endometriosis, which is an invalidating but not a life-threatening disease, side effects are less acceptable than in potentially lethal malignancies. For this reason, TNP-470 may be less suitable as a potential therapy for endometriosis although it was the most potent inhibitor of endometriosis-like lesion formation in our study. However, the large decrease in endometriosis-like

lesion formation in our study may well be caused by toxic effects of the TNP-470 on the CAM, as we observed a disturbed CAM morphology in a majority of CAMs.

Endostatin is a 20 kD C-terminal fragment of collagen XVIII. It is a potent inhibitor of *in vivo* tumour angiogenesis. In mice, systemic administration of recombinant endostatin strongly inhibited angiogenesis, maintained metastases at a microscopic size, and regressed primary tumours. No re-growth of tumour, no evidence of drug resistance and no toxicity were observed (O'Reilly *et al.*, 1997). The anti-angiogenic effect of endostatin seems to be mediated by inhibition of endothelial cell migration, as well as by induction of endothelial cell apoptosis (Dhanabal *et al.*, 1999). We showed in the CAM model for endometriosis that endostatin inhibits angiogenesis as well as endometriosis-like lesion formation.

In the search for novel anti-angiogenic agents, a β-sheet forming cytokine-like peptide, anginex, was developed (Griffioen *et al.*, 2001). Anginex acts by specifically blocking adhesion and migration of angiogenically activated endothelial cells, leading to apoptosis and ultimately to inhibition of angiogenesis *in vitro* and *in vivo*. Preliminary data indicate that anginex does not influence physiologic angiogenic responses in mice. Anginex may be a specifically acting anti-angiogenic agent for pathological angiogenesis. In the present study, anginex was used for the first time in a model for endometriosis, in which it effectively inhibited angiogenesis as well as endometriosis-like lesion formation.



For effective treatment of endometriosis by inhibition of angiogenesis, evidence is needed that not only the development of endometriosis is prevented as was shown in our study and in the study of Hull and co-workers (Hull *et al.*, 2003), but that established endometriosis will be eradicated as well. Currently, we are investigating the effects of the anti-angiogenic agents tested in this study on established endometriosis-like lesions in nude mice. Eradication of established endometriosis by these agents will be promising for future possibilities of anti-angiogenic agents for the treatment of endometriosis. Moreover, angiostatic agents should act selectively in order to make them suitable for the treatment of endometriosis. Physiological angiogenesis required in processes in the female reproductive system should not be affected. As VEGF-A is the most importantly expressed angiogenic factor in human endometrium (Charnock-Jones *et al.*, 1993), an anti-VEGF-A antibody might be suitable to inhibit endometriosis-specific angiogenesis. Anti-angiogenesis therapy will only be suitable for the treatment of endometriosis if the vasculature of the ectopically implanted endometrium can be inhibited selectively.

In conclusion, we have demonstrated that angiogenesis is a prerequisite for the development of endometriosis. Our data demonstrate that inhibitors of angiogenesis effectively interfere with the formation of endometriosis-like lesion formation. More

studies are required to investigate whether anti-angiogenic agents not only can prevent the development of endometriosis, but may eradicate established endometriosis as well. Anti-angiogenic therapy should selectively target the ectopically implanted endometrium and should not interfere with physiological angiogenesis.

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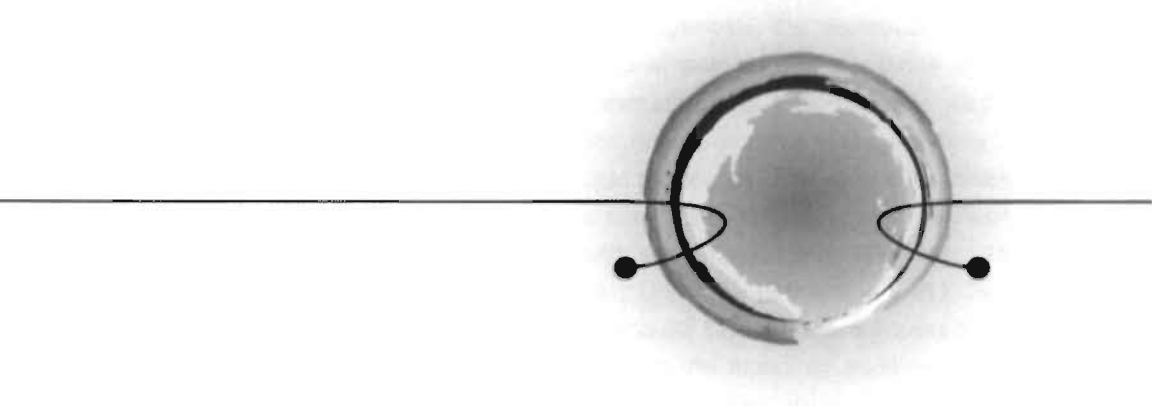
# Chapter 5

## Anti-angiogenesis therapy for endometriosis



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## Abstract

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterine cavity and is one of the most frequently encountered benign problems in gynaecology. Assuming that angiogenesis, the formation of new capillaries from pre-existing blood vessels, is of pivotal importance in the pathogenesis of endometriosis, inhibition of angiogenesis by angiostatic therapy may interfere with the development of endometriotic lesions. In the treatment of endometriosis patients, prevention of endometriotic lesion development only will not be sufficient as a therapy, but treatment options have to be developed aimed at interfering with established lesions. We evaluated the effect of the angiostatic compounds anti-hVEGF, TNP-470, endostatin, and anginex on growth of established endometriotic lesions in the nude mouse model. The angiostatic compounds significantly decreased microvessel densities and inhibited established endometriotic lesions. We confirm that human endometrium is highly angiogenic and suggest that VEGF-A is the most important angiogenesis promotory factor in both proliferative endometrium and in the biology of endometriosis. Our data demonstrate that inhibitors of angiogenesis effectively interfere with the maintenance and growth of endometriosis, which shows that endometriosis is dependent on angiogenesis. This suggests that the use of angiostatic agents may be promising as a therapy for endometriosis.

## Introduction

Endometriosis, defined as endometrium outside the uterine cavity, is found primarily in the peritoneum, the ovary and the rectovaginal septum. Women suffering from endometriosis may present with chronic pelvic pain, dysmenorrhea, dyspareunia, and subfertility. The prevalence of endometriosis in women with pelvic pain and/or subfertility is estimated between 20% and 90%, thereby being one of the most frequently encountered benign gynaecological problems (Gazvani and Templeton, 2002). Endometriosis supposedly is the result of the implantation of retrogradely shed endometrium during menstruation (Sampson, 1927). Endometrium has the capacity to adhere, attach and implant ectopically (Koks *et al.*, 1999; Maas *et al.*, 2001a). For the survival of endometrium in an ectopic location, the acquisition of an adequate blood supply is essential. Endometrium has angiogenic potential (Maas *et al.*, 2001b) and endometriotic lesions are larger in areas with a rich blood supply (Nisolle *et al.*, 1993). This suggests that angiogenesis is a prerequisite for the development of endometriosis.

Angiogenesis is a sequence of events that is fundamental to a broad array of physiological events in the body including embryogenesis, the menstrual cycle and wound healing. Angiogenesis is also involved in pathological situations such as tumour growth, atherosclerosis, chronic inflammation and endometriosis (Griffioen and Molema, 2000). The use of angiostatic agents promises to provide a new therapeutic option for some of these pathologic processes. The search for inhibitors of angiogenesis has mainly concentrated on controlling 2 of the processes involved in angiogenesis: endothelial cell (EC) growth and EC adhesion (Folkman, 1985; Molema and Griffioen, 1998; Thompson *et al.*, 1999). Targeting drugs to ECs may hold promise for treatment of endometriosis because ECs are more accessible than other cells to pharmacologic agents delivered via the blood. In addition, ECs are genetically stable and thus are not easily mutated into drug resistant variants (Molema and Griffioen, 1998).



Assuming that angiogenesis is of pivotal importance in the pathogenesis of endometriosis, angiostatic compounds may interfere with the development of endometriotic lesions, as was illustrated recently (Hull *et al.*, 2003). However, in clinical practice, women will present with established endometriosis. In order to treat women who suffer from this disease, prevention of the development of new lesions only will not be sufficient as a therapy, but treatment options have to be developed which are aimed at inhibition of maintenance and growth of established lesions. Therefore, the aim of this study was to evaluate whether the angiostatic compounds anti-human vascular endothelial growth factor-A (anti-hVEGF), TNP-470, endostatin

and anginex, acting in a broad array of angiogenic mechanisms, were effective inhibitors of established endometriotic lesions. In order to test the effect of these anti-angiogenic agents for this purpose, we used the nude mouse model. Human endometrium can be transplanted into nude mice, and endometriotic lesions which are macroscopically and microscopically similar to human endometriotic lesions come to development (Zamah *et al.*, 1984; Bruner *et al.*, 1997; Nisolle *et al.*, 2000; Grümmer *et al.*, 2001; Hull *et al.*, 2003). In our study, endometriotic lesions were allowed to establish during 3 weeks. After this period, administration of angiostatic agents was initiated. We studied the number of endometriotic lesions after 2 weeks of treatment with angiostatic agents, and evaluated microvessel densities in the lesions.

We demonstrate that angiogenesis is a prerequisite for the maintenance and growth of endometriosis, and that angiostatic compounds effectively inhibit established endometriotic lesions. This indicates that the use of angiostatic compounds may be promising as a therapy for endometriosis.

## Materials and Methods

### Endometrial tissue

Proliferative endometrium (days 5 to 11 of the menstrual cycle) was collected in 8 women having ovulatory cycles (25 to 32 days) and undergoing laparoscopy for benign conditions by transvaginal biopsy using a sampling device (Gynotec, Malden, The Netherlands). Women were 25 to 42 years of age, and indications for laparoscopy were abdominal pains, tubal testing, and sterilization. No gynaecological pathology was found in any of the endometrial biopsies. The use of human endometrium was approved by the Institutional Ethical Review Committee of the University Hospital Maastricht. All women gave written informed consent. After collection, blood was removed and endometrium was kept in serum-free DMEM/HAM's F12 culture medium. For each endometrial biopsy, Haematoxylin and Eosin (H&E) staining was performed. Tissue integrity was evaluated and the day of the menstrual cycle (cycle day) was histologically confirmed by a pathologist.



### Real-time RT-PCR

In order to investigate the angiogenic profile of proliferative human endometrium, real-time RT-PCR was performed. Total RNA was isolated from human proliferative endometrial tissue using the RNeasy RNA isolation kit (Qiagen) according to the supplier's protocol. One column DNAse treatment with the RNase-free DNAse set (Qiagen) was used to remove any genomic DNA. The purity and integrity of the

RNA was checked by gel electrophoresis according to standard procedures. One µg total RNA was reverse transcribed for 1.5 hours at 42°C with 600 U of M-MLV reverse transcriptase (Promega) in 20 µL of 1x first strand buffer (Promega), and 1 mM dNTPs in the presence of 40 U RNase inhibitor RNasin (Promega) and 0.5 µg random primers (Promega). Real-time RT-PCR was carried out in an ABI PRISM 7700® Sequence Detection System apparatus (Applied Biosystems) on 30 ng cDNA in a 25 µL volume containing 1x SYBR® Green PCR master mix (Applied Biosystems), and 500 nM of the forward and the reverse primer using the following PCR profile: 10 minutes at 95 °C, followed by 50 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Primers used for real-time RT-PCR were targeted against beta-actin (ß-Actin), cyclophillinA (cycloA), VEGF-A,-B,-C, and -D, angiopoietin 1, 2, and 3 (ANG-1, -2, -3), basic fibroblast growth factor (bFGF), placental growth factor (PlGF), VEGF-receptors 1, 2 and 3 (VEGF-R1, VEGF-R2, VEGF-R3), neuropillin 1 and 2 (NRP-1, NRP-2), and tyrosine kinase receptors 1 and 2 (Tie-1 and Tie-2). The parameter Ct (Cycle threshold) was defined as the cycle number at which the fluorescent signal passed a fixed value and the expression of each target gene was normalized to the expression of the control genes.

### Reagents

HuMV833, a humanized VEGF-A antibody (anti-hVEGF, provided by Protein Design Labs, Fremont, USA), TNP-470 (AGM-1470, provided by Takeda Chemical Industries, Osaka, Japan), endostatin (provided by Entremed Inc., Rockville, MD.), and anginex (Griffioen *et al.*, 2001) were used. As TNP-470 is a strong general angiogenesis inhibitor we used this agent as a positive control for angiostasis.



### Nude mouse model

57 female mice (Swiss nu/nu, Charles River, Maastricht, Netherlands) were individually housed in autoclaved cages and bedding, in laminar-flow filtered hoods. The animal room was maintained at 26°C with a 12-h light/12-h dark cycle, and mice were fed ad libitum with autoclaved laboratory rodent chow and acidified water. All handling was done in laminar-flow filtered hoods. A mixture of ketamin/xylazin (100 mg/kg ketamin, and 10 mg/kg xylazin, Eurovet, Bladel, Netherlands), subcutaneously injected in a volume of 0.1 ml/10 gram bodyweight was used to anaesthetize mice before invasive procedures, using sterile instruments. The Maastricht University Ethical Review Committee for Animal Experiments approved the use of mice for this study.

At the age of 5 weeks, sterile 60-day release capsules containing 18 mg 17β oestradiol (Innovative Research of America, Sarasota, FL, USA) were placed subcutaneously in the neck of each animal. According to the manufacturer's

information, capsules provide continuous release of oestradiol at serum concentrations of 150 to 250 pmol/l, in the range of physiological levels in mice during the estrous (Bronson and Desjardins, 1974). This stable physiological level of estrogen promotes the growth of the transplanted human endometrium and eliminates inter-mouse differences related to various stages of the estrous.

Four days after the insertion of the estrogen pellet, an entrance was made to the peritoneal cavity in the midline in the lower abdomen with an 18-G needle, and with the help of a pipette 10 fragments of fresh human endometrium in 200 µl of sterile phosphate buffered saline (PBS) were inoculated intraperitoneally in order to mimic the situation after retrograde menstruation in women. An other entrance was made subcutaneously through the skin in the flank and 10 fragments of fresh human endometrium were pipetted subcutaneously in order to enlarge the probability of recovery. Endometrial fragments were 1-2 $mm^3$ in size. Endometriotic lesions were equally found in both locations, suggesting that both subcutaneous and intra-peritoneal inoculation of human endometrial fragments result in formation of endometriotic lesions in nude mice.

To study normal lesion development and vascularisation in time, 2 control mice were sacrificed by cervical dislocation, 1, 2, 3 and 4 weeks after implantation of the endometrial fragments.

To study the effect of angiostatic agents on established endometriotic lesions and their vascularisation, the other mice were divided into 5 groups: a control group (n=15) and 4 groups that received anti-angiogenic agents anti-hVEGF (n=11), TNP-470 (n=4), endostatin (n=9), or anginex (n=10). Three weeks after implantation of endometrial fragments, administration of angiostatic agents was initiated. Anti-hVEGF (3 mg/kg/day) and TNP-470 (20 mg/kg every 2 days) were administered subcutaneously. Endostatin (2 mg/kg/day) and anginex (8 mg/kg/day) were administered by mini osmotic pumps (Alzet, DURECT Corporation, Cupertino, CA, USA) placed subcutaneously on the back of the animals. To control mice, 100 µl of normal saline was administered daily, subcutaneously. Five weeks after implantation of the endometrial fragments, all mice were sacrificed.



**Analysis of endometriotic lesions and vascularisation in nude mice**

To evaluate endometriotic lesions and vascularisation, the abdominal skin was opened and the abdominal subcutaneous region, the peritoneum and visceral organs were examined under a surgery microscope. Uterus and lesions with possible endometriosis were removed, fixed in 10% buffered formalin and embedded in paraffin. Paraffin sections (4 µm) were cut from the entire specimen (150-200 sections) and sections were H&E stained or used for

immunohistochemistry. Histology of endometriotic lesions was evaluated by a pathologist specialized in gynaecology and a laboratory animal pathologist.

The number of von Willebrand Factor (vWF) stained vessels (as described below) in the lesion was counted under 200x magnification, and the number of vessels per $mm^2$ lesion was calculated. Under identical circumstances, the number of mature vessels surrounded by α-smooth muscle actin (αSMA) positive cells (as described below) was calculated. To calculate the number of newly developed vessels, the number of αSMA stained vessels was subtracted from the number of vWF stained vessels. Each lesion was examined twice, and the average of the counts was taken.

### Immunostaining

Immunostaining for VEGF on human endometrium was performed using an antibody against VEGF (polyclonal, 1:200, Novocastra Laboratories LTD, Newcastle upon Tyne, UK). Human origin of endometriotic lesions was confirmed by immunostaining using an antibody against pancytokeratin (MNF 116, monoclonal, 1:500, DAKO, Glostrup, Denmark), specifically staining human epithelial cells. Blood vessels were stained by immunohistochemistry using an antibody against von Willebrand Factor (vWF) (polyclonal, 1:1000, DAKO, Glostrup, Denmark), and smooth muscle cells surrounding mature vessels were stained using an antibody against α-smooth muscle actin (αSMA)-FITC (monoclonal, 1:3000, Sigma, Saint Louis, Missouri, USA). Antibody binding was visualised with Envision (anti-VEGF, MNF 116, vWF) or with anti-FITC-HRP (αSMA) labeled secondary antibody, and 3,3'-diaminobenzidin (DAB) as a chromogen. Sections were counterstained with haematoxylin and mounted with coverslips.



### Statistical analysis

Descriptive statistics (median, range and percentiles) were calculated for each experimental group. Differences in number of endometriotic lesions as well as differences in microvessel densities between groups of mice were compared using the non-parametric Mann-Whitney-U test. P-values $<0.05$ were considered statistically significant. Microvessel densities were counted blindly, twice by 1 observer, and 1 time by a second observer under identical circumstances. The average of the counts was taken. Intraobserver and interobserver variabilities were between 5-10%.

# Results

### Human endometrial tissue is highly angiogenic

The angiogenic profile of proliferative endometrial tissue was investigated by quantitative real time RT-PCR (qRT-PCR, Figure 1a). VEGF-A was found to be the predominant angiogenic factor. This was confirmed by detection of VEGF at the protein level with immunohistochemistry (Figure 1b, 1c). qRT-PCR revealed that bFGF and angiopoietin-2 expressions were also relatively high, whereas VEGF-B and -C, PlGF and angiopoietin-1 were expressed at low levels. VEGF-D and angiopoietin-3 were virtually absent. Interestingly, whereas VEGF-R1 and -2 were expressed moderately, neuropillin-1 was the predominantly expressed angiogenesis receptor in the endometrial tissue (Figure 1a).

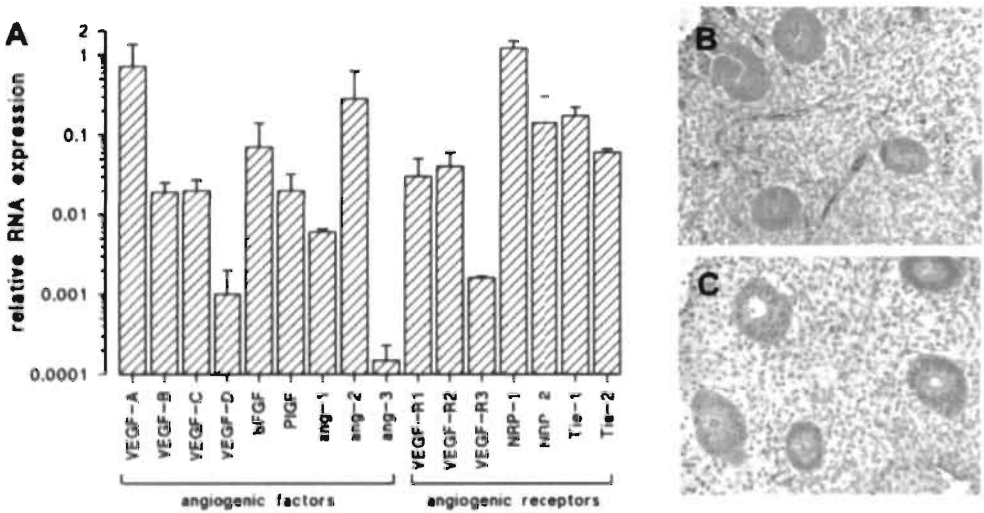


*Figure 1.* *Angiogenesis profile of human endometrium.*
*a: Quantitative real-time RT-PCR for endothelial growth factors. b: Immunostaining for human VEGF in proliferative human endometrium shows that VEGF is expressed abundantly.*
*c: Negative control staining in the same proliferative human endometrial specimen (both magnification 200x).*

### Development of endometriosis in mice is inhibited by angiostatic agents

Human endometrial fragments injected subcutaneously and in the peritoneal cavity of estrogen suppleted athymic mice gave rise to endometriotic lesions in both locations in >95% of mice. In a longitudinal study, 2 or 3 lesions were found in each mouse when mice were sacrificed 1, 2, 3 or 4 weeks after inoculation, with an

average of 2.5 lesion per mouse (not significant). This indicates that engraftment of endometrium results in quick and stable development of endometriotic lesions. In time, an increase in microvessel density was observed, when determined by vWF-staining, going up from 5 vessels/mm$^2$ lesion after 1 week (range 3-8) to 13.5 vessels/mm$^2$ lesion after 5 weeks (range 6-41, $p<0.005$).

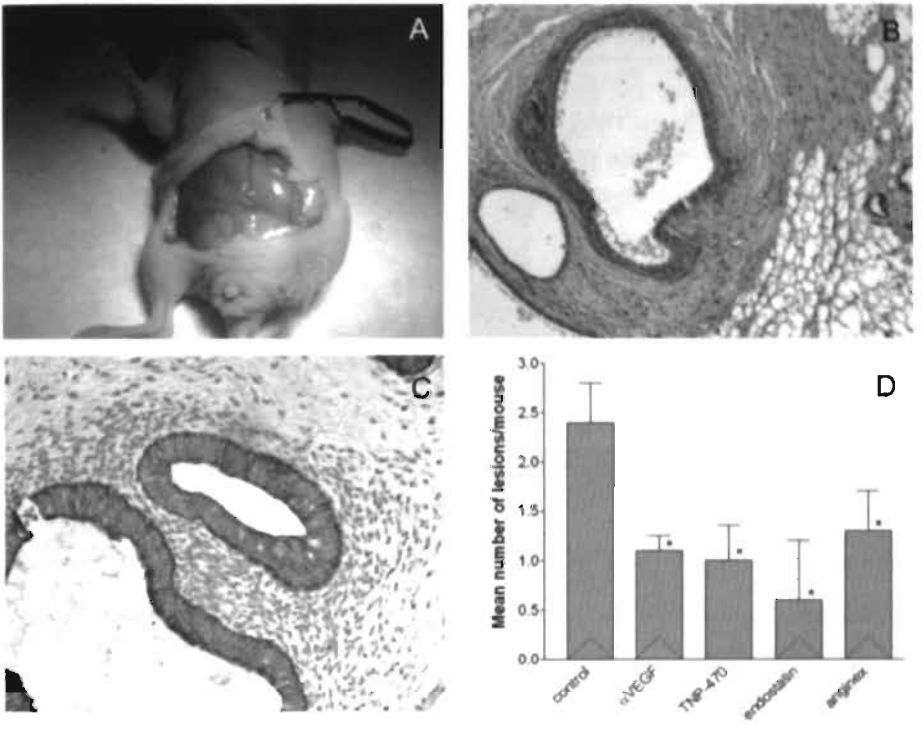


*Figure 2. Formation and morphology of endometriotic lesions in nude mice after administration of angiostatic agents.*
*a: Intraperitoneal lesion in nude mouse (control). The endometriotic lesion is visible through the peritoneum, forming white nodules in the mouse abdomen. b: Representative cross section of a subcutaneous endometriotic lesion in a nude mouse (H&E staining). Note the heterogeneous morphology of the endometrium with tubal metaplasia surrounded by fibrosis, and highly cylindrical epithelium covered with typical endometrial stroma (control mouse, magnification 200x). Panel c shows endometrial glands stained with a specific human pancytokeratin antibody (MNF 116) in a peritoneal endometriotic lesion, showing that the endometriotic lesion is of human origin (control mouse, magnification 300x). Panel d: number of endometriotic lesions in nude mice after treatment with angiostatic agents.*

*Table 1. Numbers of vWF stained vessels, of mature αSMA stained vessels, and the difference between vWF and αSMA stained vessels in endometriotic lesions in nude mice per mm² of lesion.*

| | vWF stained vessels Average (range) | αSMA stained vessels Average (range) | vWF+ αSMA- vessels Average (range) |
|---|---|---|---|
| control | 15.4 (1-52) | 9.9 (1-29) | 5.7 (0-30) |
| anti-hVEGF | 8.6 (1-18) * | 6.3 (1-17) | 2.5 (0-8) * |
| endostatin | 5.3 (3-8) * | 5.0 (2-8) | 0.0 (0) * |
| anginex | 5.9 (3-10) * | 5.4 (3-9) | 1.0 (0-9) * |

**p<0.05 compared to control.*

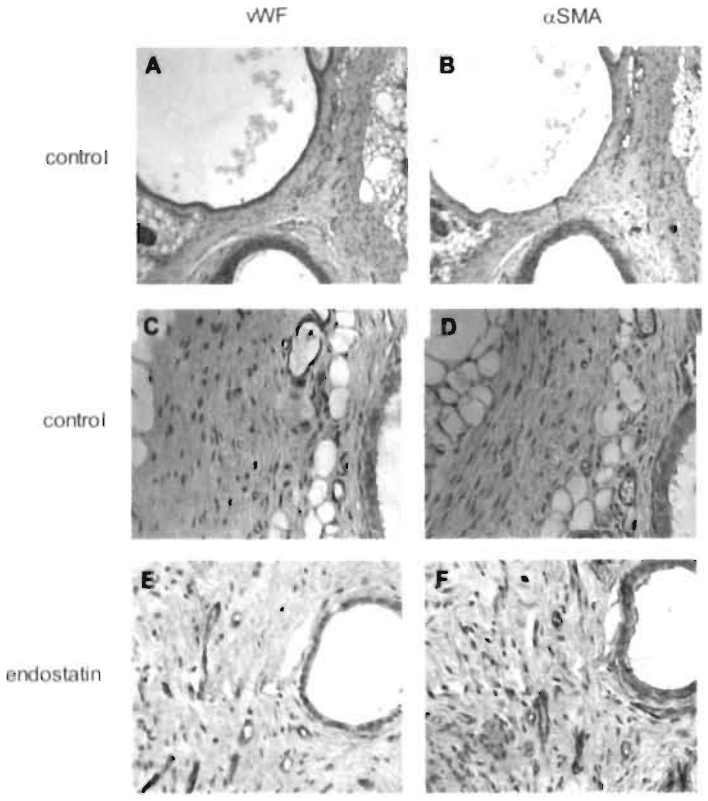


*Figure 3. vWF and αSMA staining of vessels in endometriotic lesions in nude mice. In control mice all vessels are vWF-positive (3a, 3c), but not all are αSMA-positive (3b, 3d), indicating that mature as well as newly formed vessels are present in endometriotic lesions in control mice. 3a and 3b show an overview of a representative endometriotic lesion (magnification 200x). In panel 3c and 3d detailed representative pictures are given, in which vWF-positive and αSMA-negative vessels are present (magnification 300x). In mice treated with endostatin, all vWF-positive vessels (3e) are αSMA-positive (3f), indicating that only mature vessels remain and that no new vessels come to development as a consequence of treatment with anti-angiogenic agents (magnification 300x).*

After 5 weeks, in control mice an average of 2.5 lesion was present. Treatment with anti-hVEGF resulted in a significant decrease in the number of lesions observed per mouse ($p<0.05$, Figure 2d). This indicates that VEGF is a key player in the endometrium derived signals. In addition to anti-hVEGF, mice were treated with 3 other angiogenesis inhibitors, i.e. the fungus derived antibiotic TNP-470 (Ingber *et al.*, 1990), and the specific angiogenesis inhibitors endostatin (O'Reilly *et al.*, 1997) and anginex (Griffioen *et al.*, 2001; Mayo *et al.*, 1996; Van der Schaft *et al.*, 2002). All 3 compounds significantly inhibited the number of endometriotic lesions present in mice ($p<0.05$, Figure 2d).

Morphology was assessed by a laboratory animal pathologist and by a pathologist specialized in gynaecology. A pancytokeratin staining specific for human epithelium confirmed the human origin of the endometriotic lesions (Figure 2c). All endometriotic lesions showed a heterogeneous morphology. Part of the lesions presented as tubal metaplasia (endosalpingeosis) consisting of metaplastic epithelium, surrounded by fibrotic tissue instead of stroma. This morphology equals morphology often seen in rectovaginal endometriosis in women. Other parts of the lesions contained more typical highly cylindrical endometrial epithelium surrounded by stroma (Figure 2b), as is typical for peritoneal endometriosis in women. Histology did not differ between the groups, and no relation was found between histology and vascular density of the lesion.

**Anti-angiogenic agents suppress neovascularisation in lesions in nude mice**

Microvessel density, determined on the basis of vWF positive vessels was suppressed in endometriotic lesions in mice treated with angiostatic agents ($p<0.05$, Table 1), as compared to control mice. Endostatin was found to be the most effective inhibitor of microvessel density. Both competition of VEGF and treatment with anginex led to reduction of the microvessel density. Similar results were observed for endometriotic lesions in mice treated with TNP-470, but these results were left out of Table 1 due to the low number of lesions. When vessel density was determined using SMA antibodies, quantifying mature vessels, no differences were observed between the treatment groups. Enumeration of the number of $vWF^+$ $\alpha SMA^-$ vessels, which can be considered the newly formed vessels, revealed a suppressed appearance in all groups treated with angiogenesis inhibitors ($p<0.05$, Table 1). Figure 3 shows the difference in vWF and αSMA stained vessels in an endometriotic lesion in mice.

# Discussion

The aims of the present study were to assess whether maintenance and growth of endometriotic lesions are dependent on angiogenesis and whether angiogenesis inhibition is a therapeutic possibility for endometriosis. The current data demonstrate that maintenance and growth of endometriosis are strongly dependent on angiogenic processes, and that angiostatic agents are able to inhibit established endometriotic lesions. These data were obtained from an *in vivo* human xenograft animal model using angiogenesis inhibitors for intervention.

Real time RT-PCR analysis of proliferative endometrium demonstrated that VEGF-A is the most abundantly expressed angiogenic factor in human endometrium, which suggests a pivotal role of VEGF in endometrium biology, and which confirms earlier results by others (Charnock-Jones *et al.*, 1993; Fujimoto *et al.*, 1999; Sharkey *et al.*, 2000; Taylor *et al.*, 2002; Hull *et al.*, 2003). Although the expression of VEGF was present in both epithelial and stromal cells of the endometrium, a dominant expression was observed in the microvessels within the endometrial tissue. The presence of both VEGFR-1 and -2 as well as the extremely high expression of neuropillin-1, which is a co-receptor of VEGF-A, supports the fact that VEGF-A is the most important angiogenic factor in endometrial tissue.

In a recently published study, the effect of angiogenesis inhibition was studied in nude mice. VEGF-A inhibitors were administered immediately after implantation of cultured human endometrial fragments (Hull *et al.*, 2003). The authors observed impaired lesion formation, and they concluded that angiostatic agents may be effective in the treatment of endometriosis. However, angiostatic agents were applied immediately after implantation of human endometrium, when endometriotic lesions had not yet developed. Therefore, development of endometriotic lesions was prevented in this study, but no therapy for established endometriotic lesions was applied. In our study we used uncultured human endometrium in order to avoid adverse effects. Moreover, we initiated angiostatic treatment 3 weeks after implantation of human endometrial fragments. In these 3 weeks, endometriotic lesions were allowed to establish. Start of angiostatic therapy after endometriotic lesions have established seems to be a more realistic study design, since in the clinical situation, treatment is initiated in women after endometriosis has been diagnosed, at which moment endometriotic lesions have already been present for a period of time.



We applied not only an anti-hVEGF strategy but also a number of angiostatic agents affecting a broader array of angiogenesis mechanisms. Not only anti-hVEGF, but TNP-470, endostatin and the newly developed anti-angiogenic agent anginex turned

out to effectively interfere with established endometriotic lesions as well. We found that the number of endometriotic lesions after 5 weeks of incubation was significantly lower in mice treated with angiostatic agents compared to the control group. The observed effects are ascribed to the anti-angiogenic capacity of these agents. Interestingly, physiological angiogenesis in the mice appeared not to be affected by the angiogenesis inhibitors. Surgery in mice was performed without bleeding complications later on, and no differences were observed in visual aspects of wound healing between control mice and mice treated with angiostatic agents. Moreover, the vascularisation of the uteri of mice in different groups did not differ (data not shown). These observations suggest that normal angiogenesis was not affected by the application of angiostatic agents.

The morphology of endometriotic lesions in all treatment groups was diverse, with typical endometrial glands and stroma, as well as tubal metaplasia with large cysts and flattened epithelium. The observed morphology was similar to the morphology that is often seen in endometriosis in women. We found no relation between the morphology and the vessel density of the lesions.

Vessel density, based on staining with vWF antibody was significantly lower in mice treated with angiostatic agents compared to control mice, even though the number of αSMA positive, mature vessels did not differ between the groups. This indicates that the vessels that have regressed were the newly developed ones, and not the smooth muscle cell-protected, mature vessels. Apparently, development of new blood vessels remains of pivotal importance for the maintenance and growth of endometriosis. This is also obvious from clinical observations, where newly developed, red peritoneal endometriotic lesions are vascularised by many small blood vessels with mitotically active endothelial cells (Nisolle *et al.*, 1993), and relatively small numbers of smooth muscle cell-protected adult blood vessels (Matsuzaki *et al.*, 2001). With age, the lesions evolve into black, hemorrhagic lesions, with larger blood vessels (Nisolle *et al.*, 1993), that have a higher vessel maturation index, suggesting that the number of smooth muscle cell-protected blood vessels has increased. However, unprotected vessels remain present (Matsuzaki *et al.*, 2001). Therefore, angiostatic therapy may delay the progression of established endometriosis.



To date, endometriosis is hormonally treated, aimed at achieving a hypo-estrogenic state. Hormonal therapy only suppresses symptoms, but will not eradicate the ectopic implant. Moreover, there are significant side effects. Long term hormonal therapy, therefore, is not an attractive option. Alternatively, endometriosis can be treated surgically. Conservative surgery consists of ablation of endometriotic lesions, resulting in pain relief but symptoms may recurr in time in a majority of

women. Radical surgery includes removal of the uterus and/or the ovaries, giving more permanent symptom relief, but resulting in the end of reproductive life. An effective therapeutic agent for endometriosis would be a compound that not only prevents development of endometriotic lesions, but that also would be effective against growth of established lesions. In cancer, ECs have been shown to play a pivotal role in tumour cell survival and growth. In analogy with tumour growth, endometriosis is shown to be highly dependent on angiogenesis, which makes the achievements in the field of cancer research applicable to endometriosis. Recently, breaking results have been achieved with Avastin, a humanized anti-VEGF antibody. This approach of neutralizing VEGF provided the first proof-of-concept that anti-angiogenesis is applicable in man (Ferrara, 2002; McCarthy, 2003). The major role of VEGF in endometriosis may predict the success of Avastin in endometriosis.

In conclusion, we have shown that angiogenesis is a prerequisite for the maintenance and growth of endometriosis. Our data demonstrate for the first time that different kinds of inhibitors of angiogenesis effectively interfere with established endometriotic lesions. Therefore, we favor anti-angiogenesis therapy to be put forward for clinical testing for endometriosis. When symptoms of endometriosis have been treated by hormones or by surgery, anti-angiogenic agents may be applied in order to eradicate residual and/or microscopic endometriosis.



## Acknowledgements

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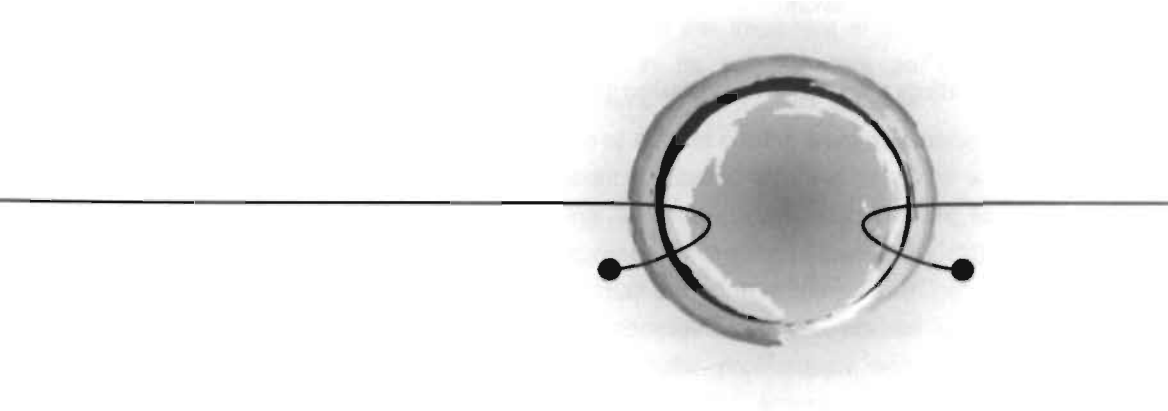
# Chapter 6

## Use of oral contraceptives prevents ectopic implantation of endometrium in the chicken chorioallantoic membrane



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Submitted

# Abstract

**Background**

Innate differences exist in the endometrium between women with and without endometriosis. Oral contraceptives (OC) may be involved in the pathogenesis of endometriosis because of their effect on the endometrium. It was hypothesized that OC use affects the ability of endometrium to implant ectopically.

**Methods**

Endometrium from women using OC and menstrual endometrium (ME) from normal cycling women was transplanted onto the chicken chorioallantoic membrane (CAM), and endometriosis-like lesion formation was evaluated. Microarray gene expression profiling was performed to identify differentially expressed genes in the endometrium from these 2 groups of women.

**Results**

Endometriosis-like lesions were formed after transplantation of OC exposed endometrium in 33% of CAMs and after transplantation of ME in 76% of CAMs ($p<0$ 05). Gene expression profiling showed a marked decrease in the expression of genes encoding for various matrix metalloproteinases, members of the transforming growth factor-β (TGF-β) superfamily and regulators of angiogenesis in OC exposed endometrium.

**Conclusion**

OC use affects the endometrium, rendering it less potent to implant ectopically.

## Introduction

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterus, predominantly within the peritoneal cavity. It presumably develops as a result of the ectopic implantation of endometrium, which has entered the peritoneal cavity via the Fallopian tubes during menstruation (Sampson, 1927). Endometriosis is one of the most commonly encountered benign problems in gynaecology, causing dysmenorrhoea, dyspareunia and chronic pelvic pain, and is associated with subfertility. Retrograde menstruation occurs to some extent in almost all cycling women, but not all of them develop symptomatic endometriosis. It has been suggested that the eutopic endometrium of women with endometriosis fundamentally differs from that of women without endometriosis in structure, proliferation, apoptosis, immune components, cell adhesion molecules, proteases and their inhibitors, steroid and cytokine production and responsiveness, gene expression and protein production (reviewed by Sharpe-Timms, 2001). Thus, innate differences in the eutopic endometrium between women may cause some to develop endometriosis whereas others do not.

Oral contraceptives (OC) are not only involved in the treatment of endometriosis-associated pain but might be instrumental in its pathogenesis as well. The endometrium of women using OC might have a diminished chance to implant ectopically as compared to the endometrium of normal cycling women. Conceivably, one explanation for this is the lower amount of tissue, which is shed by women using OC during their withdrawal bleeding. A second explanation might be the changes in the endometrium per se as a result of the exposure to the OC. The steroid hormones in OC have a different concentration and composition compared to the cyclical hormonal influence on the endometrium of the normal menstrual cycle. OC use results in an arrest of glandular proliferation and eventually endometrial atrophy. Anovulation, decidualization, amenorrhoea and the establishment of a steady estrogen-progesterone milieu may contribute to disease quiescence, and the subsequent relief of symptoms (Deligdisch, 2000; Vercellini *et al.*, 2003).



In this study, the hypothesis was tested that endometrium of women using OC has a reduced capacity to implant ectopically and form endometriotic lesions. To this end, endometrium was collected from women having normal menstrual cycles and from women using OC, and transplanted onto the chicken chorioallantoic membrane (CAM). The CAM is a suitable model to study the development of endometriosis-like lesions (Maas *et al.*, 2001; Nap *et al.*, 2003). Endometriosis-like lesion formation was evaluated in CAMs transplanted with the OC exposed endometrium and with

the ME. Furthermore, we attempted to elucidate potential mechanisms contributing to the expected difference in the potential of the endometria to implant ectopically. Gene expression profiling was performed in which differentially expressed genes in OC exposed endometrium as compared to ME were identified.

## Materials and methods

### Human endometrium

Endometrium was collected from women undergoing laparoscopy for benign conditions by transvaginal biopsy using a sampling device (Gynotec, Malden, The Netherlands). We collected endometrium from women using the monophasic oral contraceptive Microgynon® 30, containing 30 µg of ethinyloestradiol and 150 µg of levonorgestrel (Schering BV, Weesp, The Netherlands), and endometrium during the menstrual phase of the cycle (cycledays 1 to 3) from women with ovulatory cycles. In these women no endometriosis could be visualised. Ten biopsies of OC exposed endometrium and 10 biopsies of menstrual endometrium (ME) were used for the experiments in the CAM, and 2 biopsies of OC exposed endometrium and 2 biopsies of ME for the gene array analysis. The use of human endometrium was approved by the Institutional Ethical Review Committee of the University Hospital Maastricht. All women participating in the study gave their written informed consent.

Immediately after collection, blood clots were removed and the endometrium was minced in fragments of 1-2 $mm^3$ and placed in serum-free DMEM/HAM's F12 culture medium supplemented with 2 mmol/l L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B (all from Gibco Life Technologies, Breda, The Netherlands). Part of the endometrium samples was used for transplantation onto the CAM, and 2 samples were immediately snap frozen for RNA isolation. Endometrial tissue was embedded in paraffin, sectioned and stained with Haematoxylin & Eosin (H&E) for histological evaluation of the tissue integrity. The menstrual endometrium was dated according to the clinical information about the start of the last menstrual cycle.



### Chorioallantoic membrane model

Fertilised eggs of Lohman-selected White Leghorns were incubated and prepared as described previously (Nap *et al.*, 2003). Endometrium was carefully minced in fragments of 1-2 $mm^3$ and 1 fragment was transplanted onto each CAM. Endometrium exposed to OC was transplanted onto 52 CAMs, and ME was transplanted onto 86 CAMs (Table 1, Figure 1).

*Table 1* *Number of CAMs with endometriosis-like lesions related to total number of CAMs per biopsy in menstrual endometrium (ME) and in OC exposed endometrium (OC). In brackets: median and range.*

| CAMs with lesion / total number of CAMs (ME) (7.5; 4-15) | CAMs with lesion / total number of CAMs (OC) (4.5; 2-10) |
|---|---|
| 4 / 4 | 2 / 7 |
| 10 / 11 | 1 / 2 |
| 4 / 4 | 2 / 5 |
| 4 / 5 | 2 / 8 |
| 10 / 15 | 1 / 3 |
| 5 / 14 | 3 / 10 |
| 10/ 10 | 0 / 2 |
| 13 / 15 | 1 / 3 |
| 3 / 4 | 3 / 8 |
| 2 / 4 | 2 / 4 |
| Total 65 / 86 | Total 17 / 52 |

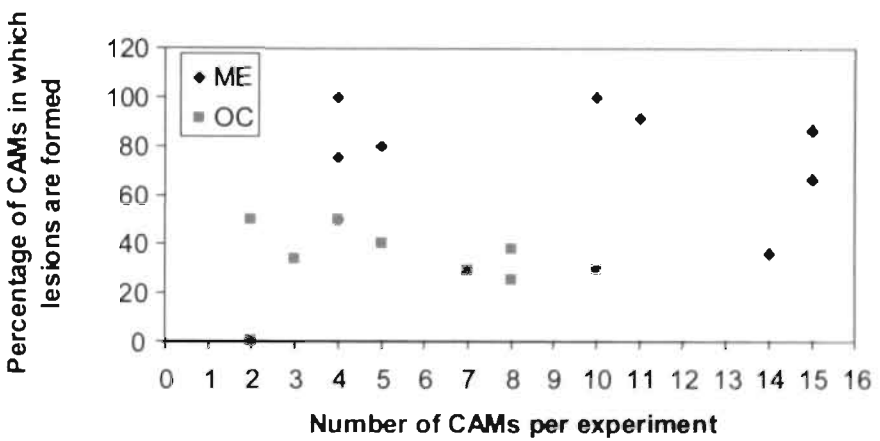


*Figure 1* *The number of CAMs transplanted with endometrium from 1 endometrial biopsy and the percentage of CAMs in which endometriosis-like lesions developed. Irrespective of the number of CAMs transplanted with endometrium from one endometrial biopsy, in all but one experiments the percentage of endometriosis-like lesions was higher in the group of the menstrual endometrium.*

CAMs with the transplanted endometrium were incubated for 72 hours. To monitor endometriosis-like lesion formation, CAMs were fixed in 3.7% buffered

formaldehyde, and the area of the CAM containing the endometrial fragment was excised and embedded in paraffin. From the entire specimen, paraffin sections (4 μm) were cut (150-200 sections). Every fifth section was stained with H&E for histological evaluation.

**RNA isolation**

Total RNA was isolated from explants using the SV total RNA isolation kit (Promega, USA) according to the manufacturer's protocol, except that the concentration of DNase-1 was doubled and the incubation time extended to 45 minutes during the DNase treatment to completely remove genomic DNA. Total RNA was eluted from the column in 50 μl RNase-free water and stored at -70$^0$C until further analysis. The quality of the RNA samples was determined by evaluating the 18S and 28S RNA bands under UV light, after agarose gel electrophoresis and ethidium bromide staining. A GAPDH PCR was performed to test for genomic DNA contamination of the isolated RNA.

**Oligonucleotide microarray analysis**

For microarray analyses we used the Affymetrix GeneChip platform employing a standard protocol for sample preparation and microarray hybridization, as detailed previously (Dürig *et al.*, 2003). Briefly, total RNA was converted into double-stranded cDNA using an oligo-deoxythymidine (oligo-dT) primer containing the T7 RNA polymerase binding site
(5'-GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAGA-$(dT)_{24}$V-3')
(MWG Biotech, Munich, Germany) for first strand synthesis. After generation of double-stranded cDNA from the first-strand cDNA, biotinylated cRNA was synthesized by *in vitro* transcription using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, New York, New York, USA). Labeled cRNA was purified on RNeasy columns (Qiagen, Hilden, Germany), fragmented and hybridized to HG-U133A microarrays (Affymetrix, Santa Clara, California, USA). The arrays were washed and stained according to the manufacturer's recommendation and finally scanned in a GeneArray scanner 2500 (Agilent, Palo Alto, California, USA).



**Microarray data processing**

Array images were processed to determine signals and detection calls (present, absent, marginal) for each probeset using the Affymetrix Microarray Suite 5.0 software (MAS 5.0; statistical algorithm). Scaling across all probe sets of a given array to an average intensity of 1000 was performed to compensate for variations in the amount and quality of the cRNA samples and other experimental variables of non-biological origin. Pairwise cross comparisons of the 2 samples of menstrual

endometrium and the samples of the OC endometrium were carried out with MAS 5.0, which calculates the signal log2 ratio (Change) and significance (Change p-value) of each change in gene expression based on a Wilcoxon ranking test. Probesets exhibiting a signal log2 ratio >1.0 and a change p-value <0.0065 or a signal log2 ratio ≤1.0 and a change p-value >0.935 (corresponding to 2-fold up- or down-regulation) were identified by filtering using the Affymetrix Data Mining Tool 3.0. Those passing these cut-offs in all 4 cross comparisons were considered as differentially expressed.

**Real-time PCR**

To generate cDNA, total RNA (1 μg) was incubated with random hexamers (1 μg/μl; Promega, Madison, WI, USA) at 70°C for 10 min. The samples were chilled on ice for 5 min. A reverse transcriptase (RT)-mix consisting of 5x RT-buffer (4 μl), 10mM dNTP mix (1μl) (Pharmacia, Uppsala, Sweden), 0.1M DTT (2 μl) (Invitrogen, Carlsbad, California, USA) and superscript II reverse transcriptase (200U/μl) (Invitrogen) was added and the samples were incubated at 42°C for 1 hour. Heating the samples at 95°C for 5 min then inactivated reverse transcriptase. The cDNA was stored at –20°C until further use. Fifty nanogram of cDNA template was used in each real-time PCR reaction.

Endometrial bleeding associated factor (EBAF) and matrix metalloproteinase (MMP)-26 primers and probes were purchased from Perkin-Elmer Applied Biosystems as pre-developed assays. Human cyclophyllin A (Hs 99999904-m1) was used as an endogenous control to normalize for the differences in the amount of total RNA added to each reaction.



All PCR reactions were performed using an ABI Prism 7700-sequence detection system (Perkin-Elmer, Boston, MA, USA). The thermal cycling conditions comprised an initial decontamination step at 50°C for 2 min, a denaturation step at 95°C for 10 min and 40 cycles at 95°C for 15 sec and 60°C for 1 min. Experiments were performed in duplicate for each sample. Quantitative values were obtained from the threshold cycle number (Ct), at which the increase in the signal associated with exponential growth of PCR products is first detected with the ABI Prism 7700 sequence detector software (Perkin-Elmer). As the targets and cyclophyllin A have similar amplification efficiencies, we used the comparative Ct method (delta delta Ct) to perform relative quantification of our target genes. For details, see user bulletin #2 for the ABI PRISM 7700 Sequence Detection System, available at: http://www.uk1.unifreiburg.de/core/facility/tagman/user_bulletin_2.pdf.

Briefly, the difference in the number of cycles, ΔCt, was determined as the difference between the target gene and cyclophyllin A within each experiment. Next,

the ΔΔCt was calculated between the treated and control samples within each experiment. The fold change was calculated as $FC=2^{-\Delta\Delta C_T}$.

### Statistical analysis

Differences between the number of endometriosis-like lesions in the CAMs transplanted with OC endometrium and these with ME were calculated using $\chi^2$ tests. P-values <0.05 were considered statistically significant.

## Results

### Morphology of human endometrium

The microscopic appearance of endometrium exposed to oral contraceptives (OC) differed from that of menstrual endometrium (ME). The morphology of the OC exposed endometrium was characterized by the presence of small glands with apical snouts (Figure 2b, arrow). The ME was heterogeneous in morphology, and endometrial degeneration was present. Glands were irregularly shaped (Figure 2). Assessment by a gynaecopathologist revealed no major morphological differences within the group of the OC exposed endometrium or within the ME group.



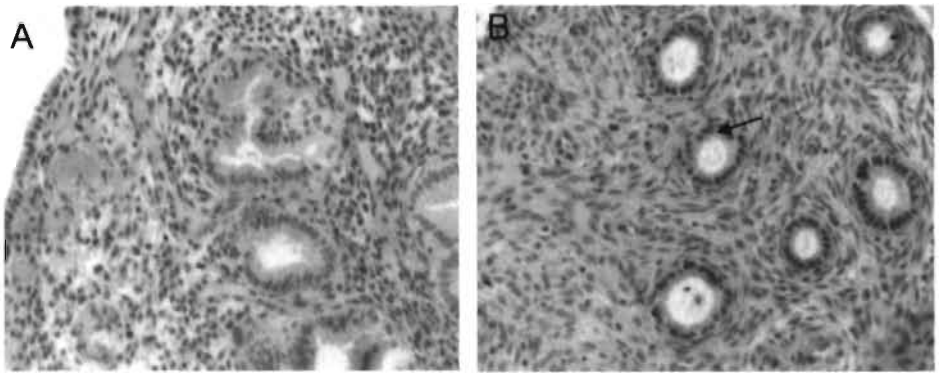


*Figure 2. The morphology of menstrual endometrium (2A) and of endometrium exposed to Oral Contraceptives (OC) (2B). H&E staining. Note the apical snouts in the OC exposed endometrium (arrow).*

### Endometrium exposed to oral contraceptives has an impaired capacity to form endometriosis-like lesions in the CAM

After seventy-two hours of incubation, endometriosis-like lesions were observed in 33% of CAMs (17 out of 52) after transplantation of OC exposed endometrium and

in 76% of CAMs (65 out of 86) after transplantation of ME ($p<0.05$; Figure 3). Table 1 and Figure 1 show the percentage of CAMs with an endometriosis-like lesion and the number of CAMs per experiment.

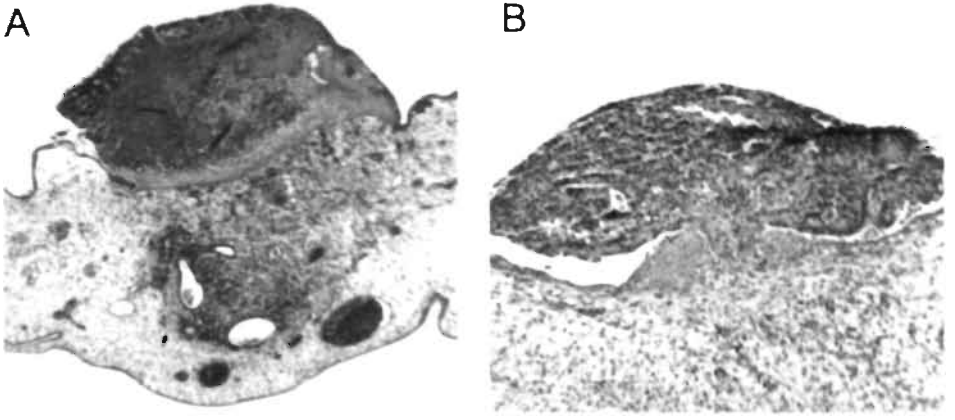


*Figure 3. Endometriosis-like lesion in CAM onto which menstrual endometrium has been transplanted (3A), and CAM without a lesion, transplanted with endometrium exposed to oral contraceptives (3B), both after 72 hours of incubation.*

### Expression profiles of endometrium exposed to oral contraceptives and of menstrual endometrium

When comparing the gene expression patterns of OC exposed endometrium to ME, a number of distinct differences emerged. Of the more than 12,000 genes analyzed, 143 gene transcripts were expressed >2-fold higher in ME, and 144 gene transcripts were expressed >2-fold lower in ME as compared to OC exposed endometrium (Appendix 2).



Table 2 presents the characteristics of 20 gene transcripts that were selected based on their relevance with regard to the potential involvement in ectopic implantation of endometrium. Inhibin βA was the gene transcript with the highest expression in ME as compared to OC exposed endometrium, with an average fold change of 177.5, whereas MMP-26 was the transcript with the lowest expression in ME, with an average fold change of 0.1.

### Real-time PCR

Two genes were selected to validate the findings of the microarray analysis with real-time PCR. These genes were endometrial bleeding associated factor (EBAF) and matrix metallloproteinase (MMP)-26. The expression of EBAF was 164-fold

higher in the ME samples, whereas the expression of MMP-26 was 7.8-fold lower in the ME as compared to the OC exposed endometrium, using real-time PCR.

*Table 2. Relative expression of gene transcripts in endometrium exposed to oral contraceptives compared to menstrual endometrium: a selection of relevant transcripts involved in ectopic implantation of human endometrium. The ratio of mRNA levels of menstrual endometrium as compared to OC exposed endometrium is expressed as average (AVG) fold change. An AVG fold change of 177.5 means that the expression is 177.5 times higher in ME as compared to OC exposed endometrium.*

| Gene Symbol | Title | AVG Fold Change |
|---|---|---|
| INHBA | inhibin, beta A | 177.5 |
| IGFBP1 | insulin-like growth factor binding protein 1 | 80.3 |
| MMP10 | matrix metalloproteinase 10 | 42.7 |
| EBAF | endometrial bleeding associated factor | 24.7 |
| BMP2 | bone morphogenetic protein2 | 14.6 |
| IL1A | interleukin 1, alpha | 9.8 |
| EDNRB | endothelin receptor type B | 8.1 |
| TIMP3 | tissue inhibitor of MMP 3 | 8.0 |
| IGFBP3 | insulin-like growth factor binding protein 3 | 6.7 |
| FGF2 | fibroblast growth factor 2 | 3.6 |
| PLAT | plasminogen activator, tissue | 3.6 |
| TGFB2 | transforming growth factor, beta 2 | 3.4 |
| THBS2 | thrombospondin 2 | 3.4 |
| ENG | endoglin | 2.9 |
| MMP27 | matrix metalloproteinase 27 | 2.8 |
| MMP2 | matrix metalloproteinase 2 | 2.8 |
| TNFAIP2 | tumour necrosis factor, alpha-induced protein 2 | 2.5 |
| EDNRA | endothelin receptor type A | 0.5 |
| IGF1 | insulin-like growth factor 1 (somatomedin C) | 0.2 |
| MMP26 | matrix metalloproteinase 26 | 0.1 |



## Discussion

For many years oral contraceptives (OC) have been used to suppress symptoms associated with endometriosis, and currently many gynaecologists consider these

drugs as the first line of therapy for the medical treatment of women with endometriosis (Winkel, 2003). As most OC have a predominant progestational effect, eventually resulting in an inactive and atrophic endometrium, women using OC shed only a small amount of endometrium during withdrawal bleeding. Conceivably, this limits the chance to develop endometriosis. Increased amounts of retrograde menstruation have been associated with a higher risk of developing the disease (Sanfilippo *et al.*, 1986; Darrow *et al.*, 1993; D'Hooghe *et al.*, 1994). We hypothesized that apart from generating less endometrium available for ectopic implantation, OC induce changes in the endometrium, making the endometrium less potent to implant ectopically. Indeed, the experiments in the chorioallantoic membrane (CAM) model demonstrate that the capacity to develop endometriosis-like lesions decreased more than 50% in the group of the OC exposed endometrium as compared to the group of the menstrual endometrium (ME). The CAM model that was used is particularly reliable to test this hypothesis, since the influence of the amount of tissue transplanted can be corrected and accounted for. The differences found are consequently not the result of the amount of tissue but of differences induced in the endometrium by the use of the hormones in the OC. In an attempt to explain the observed difference in the capacity to form endometriosis-like lesions in the CAM, microarray gene expression profiling was performed. This technique allows a hypothesis-free way to explore the molecular mechanisms of endometriosis and to identify genes abnormally expressed and possibly responsible for the development of the disease (Taylor *et al.*, 2002a).

In the present study differentially expressed genes were identified in OC exposed endometrium compared to ME, suggesting that the use of OC indeed changes the innate characteristics of the endometrium. Of the 287 differentially expressed gene transcripts, 20 genes are potentially involved in the ectopic implantation of endometrium. Strikingly, these genes were all associated directly or indirectly with either angiogenesis or with extracellular matrix (ECM) remodelling. The most markedly downregulated gene in OC exposed endometrium was inhibin βA, a member of the TGF-β superfamily. Other members of the TGF-β superfamily which were downregulated in OC exposed endometrium were EBAF, bone morphogenetic protein (BMP)-2 and TGF-β-2. Members of the TGF-β superfamily have been implicated in basically all aspects of tissue repair, including angiogenesis, the regulation of cell replication and differentiation, cellular migration, and in ECM deposition and remodelling (Godkin and Dore, 1998; Chin *et al.*, 2004). Although inhibin has been demonstrated to be present in the cystic fluid of ovarian endometriomas (Reis *et al.*, 2001), in the follicular fluid (Akande *et al.*, 2000) and in the peritoneal fluid (Florio *et al.*, 1998) of women with endometriosis, and EBAF is abundantly present in the endometrium of subfertile endometriosis patients

(Tabibzadeh *et al.*, 2000), the exact role of TGF-β in endometriosis is unclear until now. Osteen and co-workers suggested that in endometriosis, it may function as a mediator of MMP expression in concert with progesterone (Osteen *et al.*, 2003).

Besides members of the TGF-β superfamily, other angiogenic factors were downregulated in OC exposed endometrium as compared to ME. We found a low expression of angiogenic factors which have been reported to be important in endometrial angiogenesis including the insulin-like growth factor binding proteins (IGFBP) -1 and -3 (Giudice and Irwin, 1999), and thrombospondin (Seki *et al.*, 2001). A high expression of thrombospondin was associated with an angiogenic phenotype in endometrial cancer (Seki *et al.*, 2001), suggesting that thrombospondin is associated with abnormal angiogenesis. Downregulation in OC exposed endometrium was also observed of angiogenic factors supposedly involved in endometriosis, including fibroblast growth factor (FGF) (Fujimoto *et al.*, 1999), and endoglin (Kim *et al.*, 2001). FGF induces angiogenesis in endometrium together with vascular endothelial growth factor (VEGF). Endoglin interacts with members of the TGF-β superfamily, including BMP-2 (Barbara *et al.*, 1999), indicating that the different angiogenic factors reported here are acting in concert with each other.

Finally, the angiogenic factor endothelin receptor type B (EDRNB) was suppressed in OC exposed endometrium. Endothelin-1 (ET-1) acts on this receptor, thereby regulating vasoconstriction, but it also modulates tumour angiogenesis by inducing VEGF (Bagnato and Spinella, 2003). As VEGF is a predominant angiogenic factor in human endometrium (Taylor *et al.*, 2002b), appropriate regulation of this factor may be pivotal in both regulation of the menstrual cycle and in the development of endometriosis. Recent studies performed by our group confirmed that angiogenesis is essential in the development of endometriosis, and that VEGF is a major angiogenic factor in the endometrium (Nap *et al*, 2004). The current study shows that gene transcripts, which are acting in the process of angiogenesis, are downregulated in OC exposed endometrium as compared to ME, indicating that OC exposed endometrium is less angiogenic. This implies that OC exposed endometrium after entering the abdominal cavity during the withdrawal bleeding and after attachment to the peritoneal surface is less able to attract bloodvessels and hence will not survive in this ectopic location.



Gene transcripts associated with ECM remodelling are matrix metalloproteinases (MMPs) -2, -10, -27, and Tissue Inhibitor of MMP (TIMP) -3, which were all lower expressed in OC exposed endometrium as compared to ME. In previous studies in the CAM model, inhibition of MMP activity impaired endometriosis-like lesion formation (Nap *et al.*, 2004), indicating that MMPs are pivotal in the process of ectopic implantation. The lower expression of MMPs in OC exposed endometrium

may be explained by the effective inhibition of MMPs by progesterone (Osteen *et al.*, 1996). Moreover, our microarray analysis revealed a downregulation in OC exposed endometrium of IL-1 and of TNF-α-induced protein. Bruner-Tran and co-workers demonstrated that progesterone limits the effects of proinflammatory cytokines, which are potent stimulators of the MMP expression during menstruation (Bruner-Tran *et al.*, 2002). Although the cellular and molecular mechanisms by which progesterone blocks this proinflammatory cytokine action have not been elucidated, progesterone is critical to maintaining the appropriate balance between MMPs and TIMPs within the endometrium (Bruner-Tran *et al.*, 2002).

Transplantation of OC exposed endometrium still resulted in the formation of endometriosis-like lesions in 33% of CAMs. This means that mechanisms responsible for endometriosis-like lesion formation are still active. The array studies revealed that gene transcripts involved in angiogenesis including insulin-like growth factor 1 and endothelin receptor type A, as well as these encoding for the extracellular matrix mediating MMP-26 were even higher expressed in OC exposed endometrium as compared to menstrual endometrium. This may explain why endometriosis-like lesion formation still remains possible to a certain extent despite the use of OC.

The present microarray profiling study was performed with a limited number of endometrial biopsies. This carries the risk of drawing general conclusions based upon the characteristics of endometrium of individuals. This risk has been decreased as much as possible by cross comparing both biopsies in 1 group to both biopsies in the other group, and only taking into account results which were significantly different in all 4 comparisons. It has to be emphasized at this point that the relevant upregulated and downregulated genes identified in this study need to be investigated and confirmed in newly collected endometrial biopsies, to make the conclusions drawn more robust. Finally, one has to be aware of the fact that information from gene expression by its very nature does not give information on the expression on the protein level.



In conclusion, the use of OC reduces the capacity of the human endometrium to implant in an ectopic location. The use of OC causes an inhibition in the endometrium of the expression of gene transcripts which are prerequisites for the development of endometriosis, specifically those encoding for genes involved in angiogenesis and ECM remodelling. This effect is conceivably due to the progesterone component in OC. Therefore the use of OC not only diminishes pain symptoms in women with endometriosis, using OC might also be instrumental in reducing the risk to develop the disease. In future studies, the molecular mechanisms related to the findings of the present study should be investigated.

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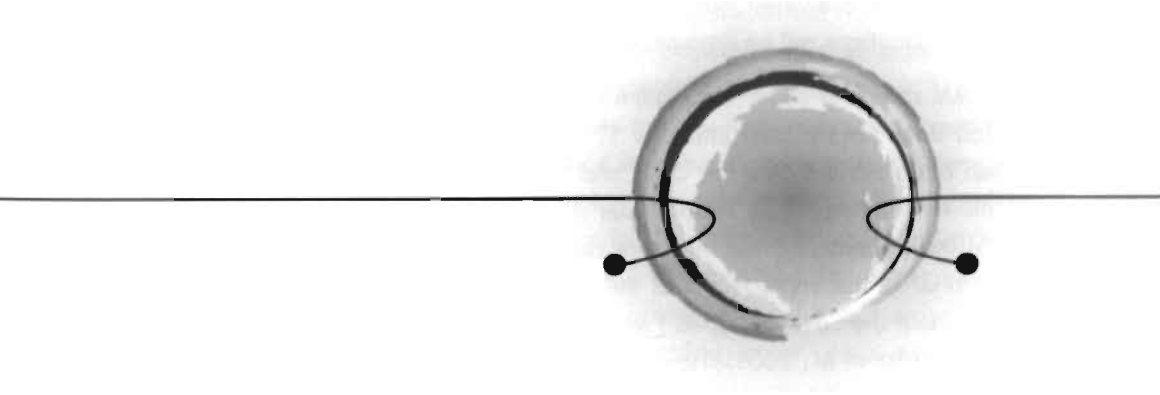
# Chapter 7

## General Discussion



# General Discussion

Of the multiple theories put forward to explain the origin of endometriosis, most evidence points to the retrograde transplantation theory. According to this theory, endometriosis in the abdomen is the result of the ectopic implantation of endometrium, which has entered the peritoneal cavity via the Fallopian tubes during menstruation (Sampson, 1927). Little information is available about the actual events occurring during the period between the moment of the arrival of the endometrial tissue in the abdomen and the first appearance of endometriotic implants. In order to implant, the endometrial fragment must be viable, adhere to the peritoneum, degrade the extracellular matrix, invade, acquire a blood supply, and survive. To gain more insight in the behaviour of endometrial tissue under ectopic conditions, we employed two model systems: the *ex vivo* chicken embryo chorioallantoic membrane (CAM) model and the *in vivo* nude mouse model. The influence of the architecture of the endometrial tissue, of the matrix metalloproteinases (MMPs), of the process of angiogenesis, and of the steroid hormone exposure of the endometrium on the initial stages of endometriotic lesion formation were evaluated individually. The results of the studies and their possible clinical implications will be discussed in this chapter.

The CAM model is easily accessible and cheap. Since the immune system of the chicken does not function until day 17 of development, it is possible to transplant human endometrium onto the CAM and to study the behaviour of this tissue in an ectopic environment. The extracellular matrix constitution of the CAM is similar to that of the peritoneum, the structure onto which the endometrium is supposed to attach prior to the implantation of the endometrial fragment. Both peritoneum and CAM contain the collagen types I and IV, laminin, and fibronectin (Giannopoulou *et al.*, 2001; Witz *et al.*, 2001). However, the CAM only partially reflects processes in the human peritoneum, because the peritoneum is exposed to hormones and local intra-abdominal environmental influences, including factors from the immune system, which are absent in the CAM. This may be considered a drawback of the CAM model. On the other hand, the lack of exposure to these local factors in the CAM does allow the study of individual processes that may contribute to lesion development without interference of the immune system.



In contrast to human amnion membranes and peritoneum that have been used to study adhesion of endometrial cells and tissue (Groothuis *et al.*, 1998; 1999), the CAM model allows the study of invasive behaviour and angiogenic potential of cells (Scher *et al.*, 1976; Armstrong *et al.*, 1982). In endometriosis research, the CAM model has mainly been used for observational studies. Our group demonstrated that

transplantation of human endometrial fragments onto the CAM induces angiogenesis and results in the formation of endometriosis-like lesions in the CAM mesenchyme (Maas *et al.*, 1999; 2001). A vascular density index (VDI) system was validated for the quantification of the number of vessels in the CAM (Maas *et al.*, 1999). Recently, the expression of angiopoietins-1 and -2 (Drenkhahn *et al.*, 2004) and MMP-1 and -2 (Wolber *et al.*, 2003) by ectopic endometrium transplanted onto the CAM has been demonstrated. In the present thesis, the CAM model has been used for intervention studies in order to investigate different mechanisms potentially involved in the development of endometriosis.

The interaction between stroma and epithelium is essential for the normal functioning of endometrial tissue (Buchanan *et al.*, 1999). Therefore, it was postulated that the endometrial tissue architecture is an important determinant of the behaviour of the endometrium during the process of implantation. In previous studies in our laboratory it was shown that single endometrial cells isolated from menstrual effluent were not invasive when transplanted on the CAM, whereas the transplantation of intact tissue fragments of human endometrium resulted in the development of endometriosis-like lesions in the CAM mesenchyme (Maas *et al.*, 2001). In the study presented in this thesis, dispersed menstrual endometrial cells did not invade the epithelium of the CAM, whereas biopsied endometrial tissue from all phases of the menstrual cycle and larger endometrial fragments manually picked from antegradely shed menstrual effluent led to the development of endometriosis-like lesions. These findings are in accordance with the observations of Sillem and co-workers who demonstrated in a cynomolgus monkey model that collagenase digestion of endometrial tissue fragments prior to transplantation reduced their ability to implant ectopically (Sillem *et al.*, 1996). We conclude that tissue integrity and architecture are important for the ectopic implantation of endometrium. This supports the clinical observation that women shedding larger, intact endometrial fragments in their menstrual effluent are more prone to develop endometriosis (Darrow *et al.*, 1993).



Immunohistochemical staining performed in the study presented in this thesis showed that almost all MMPs, which are enzymes involved in extracellular matrix degradation, are expressed in peritoneal endometriosis and endometriosis of the rectovaginal space. There is still discussion whether the MMPs in endometriosis are expressed as a cause or as a consequence of the disease, and whether they are functionally involved in the pathogenesis of endometriosis. The establishment of a functional role of MMPs is crucial to understand the relevance of the presence of these proteins in endometriotic lesions. This was evaluated in the CAM model. Immunohistochemical staining of menstrual endometrial tissue which was transplanted onto the CAM, and of the endometriosis-like lesions formed from this

endometrium revealed that there was no change in the expression level of MMPs during endometriosis-like lesion formation. Inhibiting MMP activity with an inhibitor of MMPs (metalloproteinase inhibitor, MPI) -1, -2, -3, -7 and -13 significantly reduced the number of endometriosis-like lesions that developed in the CAM. As specific inhibitors are only available for a few MMPs, an MPI has been selected which inhibits the MMPs that were most prominently expressed in the menstrual endometrium. The results of this experiment suggest that the MMPs which were inhibited by this MPI are crucial in the formation of the endometriosis-like lesions. However, in a limited number of CAMs, endometriosis-like lesions were still formed despite the administration of the MPI. This indicates that other MMPs, which were not inhibited, may also play a role in the formation of endometriosis, or, that other compensatory mechanisms are operative. Since MMPs are known to interact with, activate or inhibit each other, it is only possible to elucidate their exact role in the process of the early pathogenesis of endometriosis by selective silencing or inactivation. In animal studies, different MPIs showed promising results in inhibiting tumour progression (Zervox *et al.*, 2000; Bello *et al.*, 2002), as well as endometriotic lesion formation (Bruner *et al.*, 1997). Thus far, however, clinical results have been disappointing (reviewed in Coussens *et al.*, 2002).

In the present thesis evidence is provided to support the contention that angiogenesis is a prerequisite for the development of endometriosis-like lesions, as inhibition of angiogenesis in the CAM inhibited the vascularisation and impaired endometriosis-like lesion formation. The presence of nucleated, avian erythrocytes in the vessels in and around the endometriosis-like lesions shows that the vessels are of avian origin. Grümmer and co-workers demonstrated that the new blood vessels formed in endometriosis-like lesions in the nude mouse are of murine origin (Grümmer *et al.*, 2001). This finding is in accordance with the origin of the blood supply in malignant tumours, which is also obtained from the host environment (Taraboletti and Giavanni, 2004). Apparently, blood vessels from the surrounding tissue are feeding the endometrium in the ectopic location, and inhibiting these vessels may prevent the endometrium from surviving in an ectopic location.



The CAM is fully developed on day 10 of incubation, and from that moment onwards human endometrial tissue can be transplanted onto the CAM. Seven days later the immune system of the chick starts to function and transplanted human tissue will be rejected (Seto, 1971). Therefore, the CAM is not suitable to study growth of the endometriosis-like lesion after its initial development. In order to be able to study the role of the vasculature in endometriosis after implantation of the endometrial fragment and the development of the endometriotic lesion, a different model system had to be selected. Primates with a menstrual cycle and endometriosis, such as the cynomolgus monkey or the baboon, would be most appropriate to study. However,

the possibilities of using primates for research are limited due to ethical and financial considerations. Instead, mainly rodents have been used for the *in vivo* study of the pathogenesis of endometriosis. But as rodents do not shed endometrium, they do not develop endometriosis. Hence, endometriosis-like lesions have been induced experimentally. The immunodeficient status of nude mice makes it possible to transplant human endometrium and induce endometriosis (Zamah *et al.*, 1984; Bergqvist *et al.*, 1985). Transplanted endometrium develops into endometriosis-like lesions that have morphological characteristics of human endometriosis (Nisolle *et al.*, 2000). The nude mouse model has been validated for the study of endometriosis over extended periods of time (Grümmer *et al.*, 2001), and is therefore useful for intervention studies (Bruner *et al.*, 1997; Hull *et al.*, 2003). A possible drawback of this model is that the local chronic inflammatory response as has been described in human endometriosis is lacking in experimentally induced endometriosis in nude mice because of the deficient immunesystem.

In the study presented in this thesis, uncultured human endometrium was transplanted into nude mice, endometriotic lesions were allowed to develop and subsequently anti-angiogenic agents were administered. Part of the lesions that had developed disappeared and neo-vascularisation was impaired, indicating that established lesions are dependent on angiogenesis in order to survive. Side effects of the angiostatic agents appeared to be limited, as wound healing was normal in all mice, and the body weight as well as the weight of the uterus did not differ between the groups of mice. Moreover, vascularisation of the uterus was not impaired after the administration of anti-angiogenic agents. The results of the studies presented in this thesis regarding the interference with angiogenesis in endometriosis indicate that angiostatic therapy may be promising in order to prevent new endometriosis, to treat established endometriosis, and to prevent recurrence of the disease after surgical or hormonal treatment. Avastin, an angiostatic agent which has already been applied successfully in cancer patients (Rini *et al.*, 2004) should be considered for the treatment of endometriosis.



Endometrium that has been exposed to exogenous steroid hormones *in vivo*, e.g. by the use of oral contraceptives (OC) was shown to have a reduced capacity to form endometriosis-like lesions in the CAM. Prolonged progesterone exposure of the endometrium prior to transplantation apparently impairs endometriosis-like lesion formation. To gain more insight in the underlying mechanisms, gene expression profiles of menstrual endometrium and OC exposed endometrium were compared. Various matrix metalloproteinases and angiogenic factors were expressed at lower levels in the endometrium exposed to OC as compared to menstrual endometrium. These results support the earlier findings in this thesis that MMPs and angiogenesis are pivotal to the development of endometriosis.

In synthesis, the abundance of MMPs and VEGF in menstrual endometrial fragments facilitates implantation of these fragments and creates their bloodsupply after implantation. Steroid hormone exposure impairs the potential of human endometrium to form endometriotic lesions by affecting the expression of gene transcripts involved in matrix remodelling and angiogenesis. Based on these findings it is postulated that tissue integrity, matrix remodelling, angiogenesis and steroid hormone regulation work in concert in the complicated process of ectopic implantation of endometrium.

**Future perspectives**

Irrespective of the obvious drawbacks, we consider both the *ex vivo* CAM model and the *in vivo* nude mouse model reliable models for the study of different aspects of the development of endometriosis and for evaluation of the efficacy of new agents to treat endometriosis. Interesting agents might be metalloproteinase inhibitors (MPIs), novel angiostatic agents, selective progesterone receptor modulators (SPRMs), and non-steroidal anti-inflammatory drugs (NSAIDs), including cyclo-oxygenase-2 (COX-2) inhibitors. Agents achieving satisfying results in the CAM model and in nude mouse experiments may subsequently be tested in primate models, and eventually in clinical studies.

The study of angiostatic therapy in mice as presented in this thesis has to be extended before application in women can be considered. The effects of angiostatic agents on fertility and outcome of pregnancy should be investigated, in order to see whether these agents can be given safely to women in their fertile years. Effects of angiostatic agents on the fertility of the mice, on pregnancy duration, number of animals born, number of animals alive and development of the animals could be endpoints of this study.



The effect of *adjuvant* angiostatic therapy for established endometriotic lesions in mice that have been treated surgically or hormonally, should be investigated. Angiostatic adjuvant treatment might eventually lead to a more effective suppression, or prevention of recurrence, of endometriosis in women.

Gene expression profiling may be a promising way to identify mechanisms involved in the pathogenesis of endometriosis. Studies should be undertaken in order to confirm the results of the study in the present thesis. Prospectively collected human endometrium should be tested for the expression of the identified genes. In combination with laser capture micro-dissection, which allows selection of individual cells or groups of cells, gene expression programs may be studied during the initiation and progression of ectopic implantation of endometrium. Recent studies using gene array technology comparing endometriosis and endometrial tissue

demonstrated differentially expressed genes encoding for tumour suppression (Arimoto *et al.*, 2003) and for cell cycle regulation (Lebovic *et al.*, 2002).

Human antibodies selectively targeting angiogenic vessels may be promising for the immunotherapy of endometriosis and can help to elucidate the molecular mechanisms regulating angiogenesis in the disease. Using a phage display library on endometriosis-specific angiogenesis, antibodies may be isolated which target the vasculature of the endometriosis lesions. The selected antibodies might turn out to be useful tools for diagnostic imaging, as prognostic markers, and as therapeutic targets.

The CAM model and the nude mouse model have unique properties which can be used to investigate new questions raised by the present thesis. Complete inhibition of endometriotic lesion formation was not observed after administering angiostatic agents or MMP inhibitors, or after the transplantation of endometrium that had been exposed to steroids *in vivo*. This suggests that these mechanisms act together, or that other mechanisms may be involved. The complex way in which these mechanisms act in concert has to be investigated. This can be done by inhibiting one mechanism and study the effect of this inhibition on other mechanisms possibly involved. In addition, other mechanisms, particularly those related to chronic inflammation have to be assessed. In order to mimic the human situation as closely as possible, future studies should preferably be performed in a primate model for endometriosis.

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# Summary

Endometriosis, the presence of endometrium outside the uterus, is one of the most frequently encountered benign problems in gynaecology. Pathologists and gynaecologists have been puzzled about the phenomenon of ectopic endometrium since its first description, and until now the pathogenesis is still poorly understood. This thesis aims to shed light on different aspects of early endometriotic lesion formation.

In Chapter 1 the prevailing theories regarding the pathogenesis of endometriosis are summarized. Events in early endometriotic lesion formation are described in detail, focussing on invasion, on the role of matrix metalloproteinases (MMPs) and on angiogenesis. In addition, the hypothesis and aims of this thesis are formulated.

In Chapter 2 the influence of tissue integrity on the success of infiltration and endometriosis-like lesion formation in the chicken chorioallantoic membrane (CAM) model is described. Endometriosis-like lesion formation was evaluated after transplantation of endometrium of the menstrual and non-menstrual phase of the cycle. Biopsied endometrial fragments and fragments of shed menstrual endometrium that were larger than 1 $mm^3$ in size infiltrated the CAM and formed endometriosis-like lesions 72 hours after transplantation. Transplantation of single menstrual endometrial cells did not result in endometriosis-like lesion formation. This study suggests that an intact tissue architecture of endometrium, which consists of organized glandular epithelium and stroma, is crucial for the ability of endometrium to form lesions.

In Chapter 3 the suitability of the CAM model for evaluating expression and function of MMPs during early endometriotic lesion formation is described. The expression of MMPs in menstrual endometrium, in human endometriosis, and in endometriosis-like lesions in the CAM model was investigated using immunohistochemical staining. Almost all MMPs investigated were expressed in endometrium as well as in human endometriosis and in endometriosis-like lesions in the CAM. No significant differences were present in the expression of the majority of the MMPs investigated when comparing human endometriosis and endometriosis-like lesions in the CAM. This indicates that the MMP expression profile of human endometriosis reflects that of experimentally induced endometriosis in the CAM. Thereafter, MMP function in early lesion formation was studied by application of an inhibitor of MMP activity after transplantation of endometrium onto the CAM. The application of the MMP-inhibitor resulted in significant impaired endometriosis-like lesion formation. This study

demonstrates that the CAM model can be used to evaluate the involvement of MMPs in the development of lesions, and that MMPs are important in the pathogenesis of endometriosis.

In Chapter 4 the effect of the angiostatic agents TNP-470, anti-human VEGF, endostatin and anginex on endometriosis-like lesion development in the CAM model is described. The angiostatic agents were applied onto the CAM after transplantation of human endometrial fragments. Vascularisation of the CAMs, endometriosis-like lesion formation in CAMs and morphology of the endometriosis-like lesions were assessed 72 hours after transplantation of the tissue onto the CAM. The vascularisation of the CAMs was significantly impaired as a result of administration of angiostatic agents, as was endometriosis-like lesion formation. The percentage of necrosis in endometriosis-like lesions was significantly higher in CAMs onto which angiostatic agents were applied. This study strengthens the concept that angiogenesis is a prerequisite for the development of endometriosis lesions.

In Chapter 5 a study is presented using angiostatic agents to investigate the importance of angiogenesis in endometriotic lesions which already have developed, referred to as established endometriosis. To this end, human endometrial fragments were transplanted subcutaneously and intra-abdominally in nude mice and allowed to develop into endometriosis lesions for 3 weeks. Then the angiostatic agents were administered for a period of 2 weeks. The number of endometriotic lesions was significantly lower in mice treated with angiostatic agents. In these mice, a significantly lower number of newly formed vessels was present in the lesions compared to control mice to which no angiostatic agents had been administered. The number of mature, smooth muscle cell protected vessels did not differ between the groups, indicating that the vessels that regressed were the newly developed ones. These results suggest that angiogenesis is pivotal for the maintenance of endometriotic lesions, and that angiostatic therapy may be promising as a future treatment option for women with endometriosis.



In Chapter 6 characteristics of endometrium exposed to oral contraceptives (OC) were compared to those of menstrual endometrium. Human OC exposed endometrium and menstrual endometrial fragments were transplanted onto the CAM, and endometriosis-like lesion formation was evaluated. Exposure to OC caused an impaired capacity to develop endometriosis-like lesions in the CAM. In order to find an explanation for the different behaviour of OC exposed endometrium and menstrual endometrium after transplantation onto the CAM, microarray gene expression profiling was performed to identify differentially expressed genes. This analysis revealed a marked decrease in the expression of genes encoding for

various matrix metalloproteinases, members of the TGF-β superfamily and regulators of angiogenesis in OC exposed endometrium as compared to the expression of genes in menstrual endometrium. Conceivably, the progesterone component in OC is responsible for the effect on the endometrium, rendering it less suitable for implantation in ectopic locations.

In Chapter 7 the results of this thesis are discussed. Perspectives for future studies are given.

# Samenvatting

Endometriose, gedefinieerd als de aanwezigheid van endometrium buiten de uterus, is een van de meest voorkomende goedaardige afwijkingen in de gynaecologie. Pathologen en gynaecologen hebben zich het hoofd gebroken over het fenomeen van ectopisch endometrium sinds de eerste beschrijving ervan, maar de pathogenese van endometriose is nog niet volledig achterhaald. In dit proefschrift worden verschillende aspecten van deze pathogenese belicht.

In Hoofdstuk 1 worden de gangbare theorieën omtrent de pathogenese van endometriose samengevat. De stappen in het ontstaan van de endometrioselaesie worden in detail beschreven, waarbij de nadruk wordt gelegd op de rol van invasie, van matrix metalloproteinases (MMPs) en op die van angiogenese. Tevens worden de hypothese en doelstellingen van dit proefschrift geformuleerd.

In Hoofdstuk 2 wordt de invloed van een intacte weefselstructuur van het endometrium op het succes van infiltratie en het ontstaan van endometriose beschreven in het chorioallantoïs membraan (CAM) model van de kip. Het ontstaan van endometriose-achtige laesies werd geëvalueerd na transplantatie van endometrium van de menstruele fase en van de niet-menstruele fase van de cyclus. Gebiopteerde stukjes endometrium en fragmenten van afgescheiden menstrueel endometrium die groter waren dan 1 $mm^3$ infiltreerden het CAM en vormden endometriose-achtige laesies 72 uur na het opbrengen van het endometrium. Opbrengen van individuele cellen van menstrueel endometrium resulteerde niet in vorming van endometriose-achtige laesies. Deze studie maakt aannemelijk dat een intacte weefselstructuur van het endometrium, dat bestaat uit georganiseerd klierbuisepitheel en stroma, cruciaal is voor het vermogen van endometrium om laesies te vormen.



In Hoofdstuk 3 wordt beschreven dat het CAM model geschikt is om de expressie en functie van MMPs bij het ontstaan van endometriose te evalueren. De immunohistochemische expressie van MMPs in menstrueel endometrium, in humane endometriose en in endometriose-achtige laesies in het CAM model werd onderzocht. Bijna alle onderzochte MMPs kwamen tot expressie in endometrium, in humane endometriose en in endometriose-achtige laesies. Er waren geen significante verschillen wanneer de expressie van MMPs in humane endometriose vergeleken werd met die in endometriose-achtige laesies in het CAM. Dit wijst erop dat het MMP expressiepatroon van humane endometriose bij benadering gelijk is aan dat van experimentele endometriose. De functie van MMPs in de vroege

ontwikkeling van endometrioselaesies werd onderzocht door een MMP remmer toe te dienen na transplantatie van humaan endometrium op het CAM. De toediening van een MMP remmer resulteerde in een significante vermindering van de vorming van endometriose-achtige laesies.

Deze studie toont aan dat het CAM model geschikt is voor de evaluatie van de betrokkenheid van MMPs in de ontwikkeling van endometriose en dat MMPs van belang zijn in het ontstaan van endometriose.

In Hoofdstuk 4 wordt het effect beschreven van de angiostatische middelen TNP-470, anti-humaan VEGF, endostatine en anginex op de ontwikkeling van endometriose-achtige laesies in het CAM model. De angiostatische middelen werden toegediend na transplantatie van humane endometriumfragmenten op het CAM. De vascularisatie van de CAMs, de vorming van endometriose-achtige laesies en de morfologie van de laesies werden beoordeeld 72 uur na transplantatie van het weefsel op het CAM. De vascularisatie van de CAMs en de vorming van endometriose-achtige laesies in de CAMs was significant verminderd als gevolg van de toediening van angiostatische middelen. Necrose in de endometriose-achtige laesies was significant hoger in CAMs waaraan angiostatische middelen waren toegediend. Deze studie versterkt de eerder gevonden aanwijzingen dat angiogenese een voorwaarde is voor de ontwikkeling van endometrioselaesies.

In Hoofdstuk 5 wordt een studie gepresenteerd waarin gebruik gemaakt wordt van angiostatische middelen om het belang van angiogenese te onderzoeken in al langer bestaande endometriose. Daartoe werden humane endometrium fragmenten getransplanteerd onderhuids en in de buikholte van immunodeficiënte (naakte) muizen. Na 3 weken ontstonden endometrioselaesies. Vervolgens werden gedurende 2 weken angiostatische middelen toegediend. Het aantal endometrioselaesies was significant lager in muizen die behandeld werden met angiostatische middelen. In deze endometrioselaesies was een significant lager aantal nieuw ontwikkelde bloedvaten aanwezig vergeleken met de endometrioselaesies in muizen in de controlegroep die geen angiostatische middelen toegediend hadden gekregen. Het aantal mature, door pericyten beschermde bloedvaten was niet verschillend in de endometrioselaesies van beide groepen. Dit wijst erop dat de vaten die verdwenen de nieuw ontwikkelde vaten waren. Deze resultaten suggereren dat angiogenese essentieel is voor het voortbestaan van endometriose, en dat angiostatische therapie veelbelovend zou kunnen zijn als behandeling van endometriose.



In Hoofdstuk 6 worden eigenschappen van endometrium dat is blootgesteld aan orale anticonceptiva (pil endometrium) vergeleken met die van menstrueel endometrium. Pil endometrium en menstrueel endometrium werd getransplanteerd

op het CAM, en het ontstaan van endometriose-achtige laesies werd onderzocht. Wanneer endometrium dat is blootgesteld aan de pil werd opgebracht op het CAM, kwamen minder endometriose-achtige laesies tot ontwikkeling. Om een verklaring te vinden voor het verschillende gedrag van pil endometrium en menstrueel endometrium na transplantatie op het CAM werd microarray genexpressie analyse uitgevoerd om genen te identificeren die op verschillende wijze tot expressie komen. Deze analyse toonde aan dat er in pil endometrium een belangrijke vermindering is in de expressie van een aantal genen coderend voor verscheidene MMPs, leden van de transforming growth factor (TGF)-β familie en regulatoren van angiogenese vergeleken met menstrueel endometrium. Het is aannemelijk dat de progesteroncomponent in de pil verantwoordelijk is voor dit effect op het endometrium, waardoor het minder goed in staat is om op een ectopische plaats te implanteren.

In Hoofdstuk 7 worden de resultaten van deze thesis bediscussieerd en worden perspectieven voor toekomstige studies gegeven.

## Abbreviations

| | |
|---|---|
| ANG | angiopoietin |
| bFGF | basic fibroblast growth factor |
| BMP-2 | bone morphogenetic protein-2 |
| 17- βHSD | 17-β hydroxysteroid dehydrogenase |
| CAM | chorioallantoic membrane |
| COX | cyclooxygenase |
| Ct | cycle threshold |
| Cyclo-A | cyclophillin-A |
| DAB | diaminobenzidin |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | deoxy ribo nucleic acid |
| EBAF | endometrium bleeding associated factor |
| E-cadherin | epithelial cadherin |
| EC | endothelial cell |
| ECM | extracellular matrix |
| EDRNB | endothelin receptor type B |
| EGF | epidermal growth factor |
| ET-1 | endothelin-1 |
| GnRH | gonadotrophin releasing hormone |
| HB-EGF | heparin binding-epidermal growth factor |
| H&E | haematoxylin and eosin |
| ICAM-1 | intracellular adhesion molecule-1 |
| IGFBP-1 | insulin-like growth factor binding protein-1 |
| IL | interleukin |
| MBP | myelin basic protein |
| ME | menstrual endometrium |
| MIF | macrophage inhibitory factor |
| MMP | matrix metalloproteinase |
| MPI | metalloproteinase inhibitor |
| MT-MMP | membrane type MMP |
| MW | molecular weight |
| NK cell | natural killer cell |
| NRP | neuropillin |
| NSAID | non-steroidal anti inflammatory drug |
| OC | oral contraceptives |

| | |
|---|---|
| PAI | plasminogen activator inhibitor |
| PBS | phosphate-buffered saline |
| PDGF | platelet-derived growth factor |
| $PGE_2$ | prostaglandin $E_2$ |
| PIGF | placental growth factor |
| PI | protein inhibitor |
| PMN | polymorph nuclear neutrophils |
| qRT-PCR | quantitative reverse transcriptase polymerase chain reaction |
| RNA | ribo nucleic acid |
| SI | staining index |
| αSMA | α smooth muscle actin |
| SPRM | selective progesterone receptor modulators |
| Tie | tyrosine kinase receptor |
| TGF-β | transforming growth factor-β |
| TIMP | tissue inhibitor of MMP |
| TNF-α | tumour necrosis factor-α |
| uPA | urokinase-type plasminogen activator |
| VDI | vascular density index |
| VEGF | vascular endothelial growth factor |
| vWF | von Willebrand Factor |

*Appendix 1. Characteristics of antibodies against MMPs and TIMPs, used for immunohistochemical stainings as described in chapter 3. (Source: Oncogene, La Jolla, CA, USA).*

| MMP/ TIMP | Antibody; poly/ monoclonal | Recognizes latent/active MMP | host | clone | isotype | dilution | Incubation over night / 2 hours room temperature | Pres sure cook +/- | CAT-no |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 (mono) | Latent and active | mouse | 41-1E5 | $IgG_{2a}$ | 1:400 | On | + | IM35L |
| 2 | 3 (mono) | Latent and active | mouse | 42-5D11 | $IgG_1$ | 1:100 | On | - | IM33L |
| 3 | 2 (mono) | Latent and active | mouse | 148-IA3 | $IgG_1$ | 1:20 | On | + | IM45L |
| 7 | 2 (mono) | Active (recognizes no pro-MMP7) | mouse | 176-5F12 | $IgG_1$ | 1:100 | On | + | IM47L |
| 8 | 1 (mono) | Latent and active | mouse | 115-13D2 | $IgG_1$ | 1:20 | On | - | IM38L |
| 9 | 1 (mono) | Latent and active (latent only under reducing conditions), inhibits enzymatic activity of MMP-9 | mouse | 6-6B | $IgG_1$ | 1:50 | On | - | IM09L |
| 10 | 3 (mono) | Latent and active (under reducing conditions) | mouse | VC3 | $IgG_3$ | 1:20 | 2h RT | - | IM76 |
| 11 | 2 (mono) | Latent and active | mouse | SL3-05 | $IgG_1$ | 1:100 | On | - | IM86 |
| 13 | 1 (mono) | Latent and active | mouse | 181-15A12 | $IgG_1$ | 1:50 | On | - | IM44L |
| 14 (MT1-MMP) | 3 (mono) | 60 and 66 kDA forms of MT1-MMP | mouse | 114-6G6 | $IgG_{1\kappa}$ | 1:125 | On | - | IM42L |
| 15 (MT2-MMP) | 1 (mono) | 68 and 62 kDA forms of MT2-MMP (does not react with MT1-MMP or MT3-MMP) | mouse | 162-22G5 | $IgG_{1\kappa}$ | 1:10 | On | - | IM48L |
| 16 (MT3-MMP) | 1 (mono) | 60 kDA human MT3-MMP (does not cross-react with human MT1-MMP or MT2-MMP) | mouse | 117-10C6 | $IgG_1$ | 1:10 | 2h RT | - | IM50L |
| 23 | 1 (poly) | Inhibited by TIMP-1 | rabbit | ? | IgG | 1:20 | On | + | PC470 |
| timp 1 | 1 (mono) | TIMP-1 is found in low amounts in most cells and tissues. Reacts with MMP/TIMP-1 complexes | mouse | 7-6C1 | $IgG_1$ | 1:60 | On | - | IM32L |
| timp 3 | 1 (mono) | Recognizes glycosylated and non-glycosylated TIMP-3, no cross-reactivity seen with human TIMP-1 or TIMP-2 | mouse | 130-13H4 | $IgG_1$ | 1:50 | On | + | IM43L |

*Appendix 2 Gene transcripts significantly higher expressed in menstrual endometrium as compared to endometrium exposed to oral contraceptives.*

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| growth factors, hormones, cytokines | INHBA | inhibin, beta A (activin A, activin AB alpha polypeptide) | 177.5 |
| growth factors, hormones, cytokines | IGFBP1 | insulin-like growth factor binding protein 1 | 80.3 |
| proteases, peptidases | SERPINB3 | serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3 | 78.5 |
| matrix metalloproteinases, ECM remodeling | MMP10 | matrix metalloproteinase 10 | 42.7 |
| miscellaneous | KIAA1199 | KIAA1199 protein | 27.1 |
| metabolism | CYP24A1 | cytochrome P450, family 24, subfamily A, polypeptide 1 | 25.2 |
| growth factors, hormones, cytokines | EBAF | endometrial bleeding associated factor | 24.7 |
| immunomodulators | PAEP | progestagen-associated endometrial protein | 14.1 |
| metabolism | VNN1 | vanin 1 | 13.4 |
| metabolism | GPX3 | glutathione peroxidase 3 (plasma) | 13.3 |
| receptors, cell surface molecules | KLRC3 | killer cell lectin-like receptor subfamily C, member 3 | 10.8 |
| growth factors, hormones, cytokines | IL1A | interleukin 1, alpha | 9.8 |
| miscellaneous | DACT1 | dapper homolog 1, antagonist of beta-catenin | 9.5 |
| transporter, carrier proteins | TCN1 | transcobalamin I | 9.5 |
| growth factors, hormones, cytokines | BMP2 | bone morphogenetic protein 2 | 9.4 |
| proteases, peptidases | SERPINB3 | serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3 | 8.4 |
| receptors, cell surface molecules | EDNRB | endothelin receptor type B | 8.1 |
| transporter, carrier proteins | KCND2 | potassium voltage-gated channel, Shal-related subfamily, member 2 | 7.5 |
| receptors, cell surface molecules | T1A-2 | lung type-I cell membrane-associated glycoprotein | 7.3 |
| DNA/RNA proteins, transcription factors | SOX4 | SRY (sex determining region Y)-box 4 | 6.7 |
| growth factors, hormones, cytokines | IGFBP3 | insulin-like growth factor binding protein 3 | 6.7 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| growth factors, hormones, cytokines | NID2 | nidogen 2 | 6.6 |
| ECM/cell adhesion | EFEMP1 | EGF-containing fibulin-like extracellular matrix protein 1 | 6.2 |
| receptors, cell surface molecules | LRRC15 | leucine rich repeat containing 15 | 5.8 |
| metabolism | SOD2 | superoxide dismutase 2, mitochondrial | 5.6 |
| miscellaneous | LOC51334 | mesenchymal stem cell protein DSC54 | 5.5 |
| ion-binding proteins, metal-ion regulators | FLJ13612 | likely ortholog of neuronally expressed calcium binding protein | 5.2 |
| metabolism | SDR1 | short-chain dehydrogenase/reductase 1 | 5.1 |
| matrix metalloproteinases, ECM remodeling | TIMP3 | tissue inhibitor of metalloproteinase 3 | 4.8 |
| receptors, cell surface molecules | KCNK3 | potassium channel, subfamily K, member 3 | 4.7 |
| proteases, peptidases | SPINK1 | serine protease inhibitor, Kazal type 1 | 4.7 |
| DNA/RNA proteins, transcription factors | TWIST1 | twist homolog 1 | 4.6 |
| metabolism | CYP3A5 | cytochrome P450, family 3, subfamily A, polypeptide 5 | 4.5 |
| signal transduction | --- | Homo sapiens mRNA full length insert cDNA clone EUROIMAGE 1630957 | 4.3 |
| ECM/cell adhesion | ASPN | asporin (LRR class 1) | 4.3 |
| cell cycle, proliferation, apoptosis | G0S2 | putative lymphocyte G0/G1 switch gene | 4.3 |
| transporter, carrier proteins | ABCA6 | ATP-binding cassette, sub-family A (ABC1), member 6 | 4.3 |
| receptors, cell surface molecules | CLECSF2 | C-type lectin, superfamily member 2 | 4.2 |
| transporter, carrier proteins | SLC12A8 | solute carrier family 12, member 8 | 4.1 |
| ECM/cell adhesion | LAMB3 | laminin, beta 3 | 4.1 |
| receptors, cell surface molecules | ELTD1 | EGF, latrophilin and seven transmembrane domain containing 1 | 4.1 |
| DNA/RNA proteins, transcription factors | HOP | homeodomain-only protein | 4.1 |
| miscellaneous | --- | --- | 4.0 |
| receptors, cell surface molecules | KDR | kinase insert domain receptor | 4.0 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| immunomodulators | KIR3DL2 | killer cell immunoglobulin-like receptor | 3.9 |
| metabolism | PRDM1 | PR domain containing 1 | 3.8 |
| cell cycle, proliferation, apoptosis | CLK1 | CDC-like kinase 1 | 3.8 |
| growth factors, hormones, cytokines | FGF2 | fibroblast growth factor 2 (basic) | 3.6 |
| signal transduction | MAP3K4 | mitogen-activated protein kinase kinase kinase 4 | 3.6 |
| proteases, peptidases | SERPINE2 | serine (or cysteine) proteinase inhibitor, clade E | 3.6 |
| proteases, peptidases | PLAT | plasminogen activator, tissue | 3.6 |
| DNA/RNA proteins, transcription factors | BACH2 | BTB and CNC homology 1, basic leucine zipper transcription factor 2 | 3.5 |
| ECM/cell adhesion | HABP2 | hyaluronan binding protein 2 | 3.5 |
| proteases, peptidases | TFPI | tissue factor pathway inhibitor | 3.4 |
| miscellaneous | MGC14376 | hypothetical protein MGC14376 | 3.4 |
| metabolism | NOX4 | NADPH oxidase 4 | 3.4 |
| miscellaneous | --- | --- | 3.4 |
| ECM/cell adhesion | THBS2 | thrombospondin 2 | 3.4 |
| growth factors, hormones, cytokines | TGFB2 | transforming growth factor, beta 2 | 3.4 |
| receptors, cell surface molecules | EMP1 | epithelial membrane protein 1 | 3.3 |
| growth factors, hormones, cytokines | LIF | leukemia inhibitory factor | 3.3 |
| transporter, carrier proteins | SLC22A4 | solute carrier family 22, member 4 | 3.2 |
| signal transduction | AXL | AXL receptor tyrosine kinase | 3.2 |
| DNA/RNA proteins, transcription factors | SFRS6 | splicing factor, arginine/serine-rich 6 | 3.1 |
| metabolism | FADS3 | fatty acid desaturase 3 | 3.1 |
| miscellaneous | ANKRD10 | ankyrin repeat domain 10 | 3.0 |
| receptors, cell surface molecules | TM4SF1 | transmembrane 4 superfamily member 1 | 3.0 |
| signal transduction | PKIG | protein kinase inhibitor gamma | 3.0 |
| signal transduction | CBLB | Cas-Br-M (murine) ecotropic retroviral transforming sequence b | 3.0 |
| receptors, cell surface | TM7SF1 | transmembrane 7 superfamily member 1 | 3.0 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| molecules | | | |
| ECM/cell adhesion | ENG | endoglin | 2.9 |
| ECM/cell adhesion | GALNACT-2 | chondroitin sulfate GalNAcT-2 | 2.9 |
| miscellaneous | --- | Homo sapiens cDNA clone IMAGE:4152985 | 2.9 |
| metabolism | KIAA0934 | KIAA0934 protein | 2.9 |
| signal transduction | RIN2 | Ras and Rab interactor 2 | 2.8 |
| matrix metalloproteinases, ECM remodeling | MMP27 | matrix metalloproteinase 27 | 2.8 |
| matrix metalloproteinases, ECM remodeling | MMP2 | matrix metalloproteinase 2 | 2.8 |
| cell cycle, proliferation, apoptosis | RARRES1 | retinoic acid receptor responder | 2.8 |
| ECM/cell adhesion | CLDN10 | claudin 10 | 2.7 |
| receptors, cell surface molecules | KIR2DL3 | killer cell immunoglobulin-like receptor | 2.7 |
| transporter, carrier proteins | SLC38A2 | solute carrier family 38, member 2 | 2.7 |
| receptors, cell surface molecules | GPR116 | G protein-coupled receptor 116 | 2.7 |
| metabolism | ABP1 | amiloride binding protein 1 | 2.7 |
| DNA/RNA proteins, transcription factors | ZNF216 | zinc finger protein 216 | 2.7 |
| miscellaneous | GAB2 | GRB2-associated binding protein 2 | 2.7 |
| DNA/RNA proteins, transcription factors | TIA1 | TIA1 cytotoxic granule-associated RNA binding protein | 2.6 |
| receptors, cell surface molecules | MMD | monocyte to macrophage differentiation-associated | 2.6 |
| receptors, cell surface molecules | MUC16 | mucin 16 | 2.6 |
| signal transduction | FGR | Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog | 2.6 |
| DNA/RNA proteins, transcription factors | MRPS6 | mitochondrial ribosomal protein S6 | 2.6 |
| immunomodulators | C1QR1 | complement component 1, q subcomponent, receptor 1 | 2.6 |
| DNA/RNA proteins, transcription factors | TBX3 | T-box 3 (ulnar mammary syndrome) | 2.6 |
| receptors, cell surface | GPNMB | glycoprotein (transmembrane) nmb | 2.6 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| molecules | | | |
| growth factors, hormones, cytokines | TNFAIP2 | tumour necrosis factor, alpha-induced protein 2 | 2.5 |
| signal transduction | STK38L | serine/threonine kinase 38 like | 2.5 |
| signal transduction | SSB1 | SPRY domain-containing SOCS box protein SSB-1 | 2.5 |
| immunomodulators | C1QTNF3 | C1q and tumour necrosis factor related protein 3 | 2.5 |
| signal transduction | SLA | Src-like-adaptor | 2.5 |
| cell cycle, proliferation, apoptosis | PAWR | PRKC, apoptosis, WT1, regulator | 2.5 |
| metabolism | DIO2 | deiodinase, iodothyronine, type II | 2.5 |
| immunomodulators | IFRD1 | interferon-related developmental regulator 1 | 2.5 |
| transporter, carrier proteins | SLC3A2 | solute carrier family 3 | 2.5 |
| miscellaneous | RTN3 | reticulon 3 | 2.5 |
| ECM/cell adhesion | SPP1 | secreted phosphoprotein 1 | 2.4 |
| proteases, peptidases | PRSS11 | protease, serine, 11 (IGF binding) | 2.4 |
| transporter, carrier proteins | SLC20A1 | solute carrier family 20, member 1 | 2.4 |
| DNA/RNA proteins, transcription factors | ZFP36L1 | zinc finger protein 36, C3H type-like 1 | 2.3 |
| receptors, cell surface molecules | CMRF-35H | leukocyte membrane antigen | 2.3 |
| miscellaneous | KIAA0692 | KIAA0692 protein | 2.3 |
| signal transduction | RIS | Ras family member Ris | 2.3 |
| DNA/RNA proteins, transcription factors | CEBPB | CCAAT/enhancer binding protein (C/EBP), beta | 2.3 |
| DNA/RNA proteins, transcription factors | HLX1 | H2.0-like homeo box 1 (Drosophila) | 2.3 |
| receptors, cell surface molecules | LEPR | leptin receptor | 2.3 |
| growth factors, hormones, cytokines | PRL | prolactin | 2.3 |
| signal transduction | PRKAR2B | protein kinase, cAMP-dependent, regulatory, type II, beta | 2.3 |
| DNA/RNA proteins, transcription factors | RUNX1 | runt-related transcription factor 1 | 2.3 |
| ECM/cell adhesion | FBLN5 | fibulin 5 | 2.3 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| transporter, carrier proteins | DNAJB9 | DnaJ (Hsp40) homolog, subfamily B, member 9 | 2.3 |
| metabolism | CHST6 | carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6 | 2.3 |
| signal transduction | LCP2 | lymphocyte cytosolic protein 2 | 2.2 |
| metabolism | LOC123803 | N-terminal Asn amidase | 2.2 |
| transporter, carrier proteins | RBP1 | retinol binding protein 1, cellular | 2.2 |
| miscellaneous | KIAA0379 | KIAA0379 protein | 2.2 |
| transporter, carrier proteins | SLC4A7 | solute carrier family 4, sodium bicarbonate cotransporter, member 7 | 2.2 |
| signal transduction | PIK3CD | phosphoinositide-3-kinase, catalytic, delta polypeptide | 2.2 |
| signal transduction | RAB31 | RAB31, member RAS oncogene family | 2.2 |
| miscellaneous | NUPL1 | nucleoporin like 1 | 2.2 |
| receptors, cell surface molecules | VWF | von Willebrand factor | 2.2 |
| signal transduction | SOCS5 | suppressor of cytokine signaling 5 | 2.2 |
| miscellaneous | SDCCAG8 | serologically defined colon cancer antigen 8 | 2.2 |
| transporter, carrier proteins | DDEF2 | development and differentiation enhancing factor 2 | 2.1 |
| miscellaneous | C14orf116 | chromosome 14 open reading frame 116 | 2.1 |
| signal transduction | SPRY2 | sprouty homolog 2 (Drosophila) | 2.1 |
| transporter, carrier proteins | SLC19A2 | solute carrier family 19, member 2 | 2.1 |
| receptors, cell surface molecules | HOMER1 | homer homolog 1 (Drosophila) | 2.1 |
| DNA/RNA proteins, transcription factors | CHES1 | checkpoint suppressor 1 | 2.0 |
| cytoskeleton, structural proteins | WASPIP | Wiskott-Aldrich syndrome protein interacting protein | 2.0 |
| miscellaneous | ELOVL4 | elongation of very long chain fatty acids-like 4 | 2.0 |
| signal transduction | MAP4K4 | mitogen-activated protein kinase 4 | 2.0 |
| DNA/RNA proteins, transcription factors | HBP1 | HMG-box transcription factor 1 | 2.0 |
| receptors, cell surface molecules | EPB41L3 | erythrocyte membrane protein band 4.1-like 3 | 2.0 |
| DNA/RNA proteins, transcription factors | CUGBP2 | CUG triplet repeat, RNA binding protein 2 | 2.0 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| signal transduction | RRAS2 | related RAS viral (r-ras) oncogene homolog 2 | 2.0 |
| transporter, carrier proteins | ENSA | endosulfine alpha | 0.5 |
| ECM/cell adhesion | CLDN3 | claudin 3 | 0.5 |
| metabolism | WBSCR22 | Williams Beuren syndrome chromosome region 22 | 0.5 |
| transporter, carrier proteins | FRDA | Friedreich ataxia | 0.5 |
| immunomodulators | IFI35 | interferon-induced protein 35 | 0.5 |
| proteases, peptidases | MIPEP | mitochondrial intermediate peptidase | 0.5 |
| metabolism | KIAA1698 | KIAA1698 protein | 0.5 |
| signal transduction | LIM | LIM protein | 0.5 |
| ECM/cell adhesion | EVA1 | epithelial V-like antigen 1 | 0.5 |
| signal transduction | FLJ10986 | hypothetical protein FLJ10986 | 0.5 |
| metabolism | MRPS11 | mitochondrial ribosomal protein S11 | 0.5 |
| metabolism | FKBP4 | FK506 binding protein 4, 59kDa | 0.5 |
| signal transduction | SYNJ2BP | synaptojanin 2 binding protein | 0.5 |
| metabolism | PECI | peroxisomal D3,D2-enoyl-CoA isomerase | 0.5 |
| miscellaneous | ANKS1 | ankyrin repeat and SAM domain containing 1 | 0.5 |
| receptors, cell surface molecules | LGALS3BP | lectin, galactoside-binding, soluble, 3 binding protein | 0.5 |
| proteases, peptidases | UQCRC2 | ubiquinol-cytochrome c reductase core protein II | 0.5 |
| metabolism | MTHFD1 | methylenetetrahydrofolate dehydrogenase | 0.5 |
| miscellaneous | NET1 | neuroepithelial cell transforming gene 1 | 0.5 |
| metabolism | C11orf8 | chromosome 11 open reading frame 8 | 0.5 |
| metabolism | ALAD | aminolevulinate, delta-, dehydratase | 0.5 |
| DNA/RNA proteins, transcription factors | HOXA11 | homeo box A11 | 0.5 |
| DNA/RNA proteins, transcription factors | POP5 | RNase MRP/RNase P protein-like | 0.5 |
| DNA/RNA proteins, transcription factors | NBS1 | Nijmegen breakage syndrome 1 | 0.5 |
| DNA/RNA proteins, transcription factors | DKFZP564M182 | DKFZP564M182 protein | 0.5 |
| DNA/RNA proteins, transcription factors | MSX1 | msh homeo box homolog 1 (Drosophila) | 0.5 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| DNA/RNA proteins, transcription factors | NFIB | nuclear factor I/B | 0.5 |
| metabolism | GNS | glucosamine (N-acetyl)-6-sulfatase | 0.5 |
| miscellaneous | ISG20 | interferon stimulated gene 20kDa | 0.5 |
| metabolism | GSR | glutathione reductase | 0.5 |
| receptors, cell surface molecules | EDNRA | endothelin receptor type A | 0.5 |
| miscellaneous | RUVBL2 | RuvB-like 2 (E. coli) | 0.5 |
| miscellaneous | SMT3H1 | SMT3 suppressor of mif two 3 homolog 1 (yeast) | 0.5 |
| miscellaneous | --- | Homo sapiens cDNA FLJ31439 fis, clone NT2NE2000707. | 0.5 |
| DNA/RNA proteins, transcription factors | SNRPD1 | small nuclear ribonucleoprotein D1 polypeptide 16kDa | 0.5 |
| DNA/RNA proteins, transcription factors | HNRPC | heterogeneous nuclear ribonucleoprotein C (C1/C2) | 0.5 |
| signal transduction | RABL2B | RAB, member of RAS oncogene family-like 2B | 0.5 |
| receptors, cell surface molecules | CGI-51 | CGI-51 protein | 0.5 |
| metabolism | NDUFA7 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex | 0.5 |
| miscellaneous | TOR3A | torsin family 3, member A | 0.5 |
| ion-binding proteins, metal-ion regulators | TRIM14 | tripartite motif-containing 14 | 0.5 |
| transporter, carrier proteins | PAI-RBP1 | PAI-1 mRNA-binding protein | 0.5 |
| DNA/RNA proteins, transcription factors | SMARCA2 | SWI/SNF related, matrix ass., actin dependent regulator chromatin | 0.5 |
| transporter, carrier proteins | KPNA2 | karyopherin alpha 2 (RAG cohort 1, importin alpha 1) | 0.5 |
| signal transduction | NME1 | non-metastatic cells 1, protein (NM23A) expressed in | 0.5 |
| DNA/RNA proteins, transcription factors | HNRPH1 | heterogeneous nuclear ribonucleoprotein H1 (H) | 0.5 |
| metabolism | HMGCR | 3-hydroxy-3-methylglutaryl-Coenzyme A reductase | 0.5 |
| growth factors, hormones, cytokines | PTN | pleiotrophin | 0.5 |
| DNA/RNA proteins, transcription factors | DZIP1 | zinc finger DAZ interacting protein 1 | 0.5 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| miscellaneous | DKFZP547E1010 | DKFZP547E1010 protein | 0.5 |
| DNA/RNA proteins, transcription factors | SAP18 | sin3-associated polypeptide, 18kDa | 0.5 |
| metabolism | PMVK | phosphomevalonate kinase | 0.5 |
| metabolism | MRPL17 | mitochondrial ribosomal protein L17 | 0.5 |
| vasoactive substances | SPARCL1 | SPARC-like 1 (mast9, hevin) | 0.5 |
| miscellaneous | --- | Homo sapiens LOC347499 mRNA | 0.5 |
| metabolism | FLJ22222 | hypothetical protein FLJ22222 | 0.5 |
| receptors, cell surface molecules | CD24 | CD24 antigen | 0.5 |
| cell cycle, proliferation, apoptosis | PHB | prohibitin | 0.5 |
| cytoskeleton, structural proteins | TPM1 | tropomyosin 1 (alpha) | 0.5 |
| metabolism | QDPR | quinoid dihydropteridine reductase | 0.5 |
| DNA/RNA proteins, transcription factors | POLE3 | polymerase (DNA directed), epsilon 3 | 0.5 |
| metabolism | ACLY | ATP citrate lyase | 0.5 |
| receptors, cell surface molecules | THY1 | Thy-1 cell surface antigen | 0.5 |
| metabolism | DCI | dodecenoyl-Coenzyme A delta isomerase | 0.5 |
| signal transduction | CKB | creatine kinase, brain | 0.5 |
| metabolism | SORD | sorbitol dehydrogenase | 0.5 |
| signal transduction | GNAL | guanine nucleotide binding protein (G protein) | 0.5 |
| miscellaneous | MGC4825 | hypothetical protein MGC4825 | 0.5 |
| proteases, peptidases | PSMB9 | proteasome subunit, beta type | 0.5 |
| DNA/RNA proteins, transcription factors | MED6 | mediator of RNA polymerase II transcription, subunit 6 homolog | 0.4 |
| transporter, carrier proteins | SLC15A2 | solute carrier family 15 (H+/peptide transporter), member 2 | 0.4 |
| proteases, peptidases | PSME2 | proteasome activator subunit 2 (PA28 beta) | 0.4 |
| miscellaneous | ZFYVE21 | zinc finger, FYVE domain containing 21 | 0.4 |
| metabolism | CYB5 | cytochrome b-5 | 0.4 |
| metabolism | FDPS | farnesyl diphosphate synthase | 0.4 |
| metabolism | PPIC | peptidylprolyl isomerase C | 0.4 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| cell cycle, proliferation, apoptosis | CETN2 | centrin, EF-hand protein, 2 | 0.4 |
| transporter, carrier proteins | FKBP11 | FK506 binding protein 11, 19 kDa | 0.4 |
| receptors, cell surface molecules | GPR49 | G protein-coupled receptor 49 | 0.4 |
| ECM/cell adhesion | ITGA6 | integrin, alpha 6 | 0.4 |
| transporter, carrier proteins | ETFB | electron-transfer-flavoprotein, beta polypeptide | 0.4 |
| cytoskeleton, structural proteins | ACTA2 | actin, alpha 2 | 0.4 |
| DNA/RNA proteins, transcription factors | HOXA10 | homeo box A10 | 0.4 |
| metabolism | GLA | galactosidase, alpha | 0.4 |
| miscellaneous | BITE | p10-binding protein | 0.4 |
| miscellaneous | FAIM | Fas apoptotic inhibitory molecule | 0.4 |
| receptors, cell surface molecules | FOLR1 | folate receptor 1 | 0.4 |
| ECM/cell adhesion | COL16A1 | collagen, type XVI, alpha 1 | 0.4 |
| transporter, carrier proteins | HSPB1 | heat shock 27kDa protein 1 | 0.4 |
| miscellaneous | KIAA0819 | KIAA0819 protein | 0.4 |
| metabolism | PCCA | propionyl Coenzyme A carboxylase, alpha polypeptide | 0.4 |
| miscellaneous | TUWD12 | TUWD12 | 0.4 |
| transporter, carrier proteins | STX18 | syntaxin 18 | 0.4 |
| DNA/RNA proteins, transcription factors | LSM5 | LSM5 homolog, U6 small nuclear RNA associated | 0.4 |
| metabolism | MOXD1 | monooxygenase, DBH-like 1 | 0.4 |
| transporter, carrier proteins | ATP5G1 | ATP synthase, H+ transporting, mitochondrial F0 complex | 0.4 |
| proteases, peptidases | EYA2 | eyes absent homolog 2 (Drosophila) | 0.4 |
| signal transduction | C14orf159 | chromosome 14 open reading frame 159 | 0.4 |
| cell cycle, proliferation, apoptosis | PDCD8 | programmed cell death 8 (apoptosis-inducing factor) | 0.4 |
| receptors, cell surface molecules | PGRMC1 | progesterone receptor membrane component 1 | 0.4 |
| miscellaneous | HHLA3 | HERV-H LTR-associating 3 | 0.4 |
| miscellaneous | --- | H.sapiens transcr seq with strong similarity to | 0.4 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| | | protein pir:A32800 | |
| signal transduction | PLCE1 | phospholipase C, epsilon 1 | 0.4 |
| miscellaneous | --- | H. sapiens transcr sequence, moderate simil. to prot. ref:NP_071431.1 | 0.4 |
| metabolism | C12orf8 | chromosome 12 open reading frame 8 | 0.4 |
| DNA/RNA proteins, transcription factors | RUVBL1 | RuvB-like 1 | 0.4 |
| metabolism | PPID | peptidylprolyl isomerase D | 0.4 |
| cell cycle, proliferation, apoptosis | RCL | putative c-Myc-responsive | 0.4 |
| ion-binding proteins, metal-ion regulators | SELENBP1 | selenium binding protein 1 | 0.4 |
| miscellaneous | FLJ20366 | hypothetical protein FLJ20366 | 0.4 |
| signal transduction | GRP58 | glucose regulated protein, 58kDa | 0.4 |
| metabolism | SULT1C1 | sulfotransferase family, cytosolic, 1C, member 1 | 0.4 |
| metabolism | QRSL1 | glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1 | 0.4 |
| miscellaneous | HNOEL-iso | HNOEL-iso protein | 0.3 |
| transporter, carrier proteins | ATP6V1A | ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A | 0.3 |
| signal transduction | MAP2K6 | mitogen-activated protein kinase kinase 6 | 0.3 |
| cytoskeleton, structural proteins | NEFH | neurofilament, heavy polypeptide 200kDa | 0.3 |
| DNA/RNA proteins, transcription factors | MID1 | midline 1 | 0.3 |
| cell cycle, proliferation, apoptosis | MNS1 | meiosis-specific nuclear structural protein 1 | 0.3 |
| metabolism | NQO1 | NAD(P)H dehydrogenase, quinone 1 | 0.3 |
| immunomodulators | IFI27 | interferon, alpha-inducible protein 27 | 0.3 |
| proteases, peptidases | ARTS-1 | type 1 TNF receptor shedding aminopeptidase regulator | 0.3 |
| proteases, peptidases | PLA2G4A | phospholipase A2, group IVA | 0.3 |
| miscellaneous | STXBP6 | syntaxin binding protein 6 | 0.3 |
| metabolism | ASRGL1 | asparaginase like 1 | 0.3 |
| ion-binding proteins, metal-ion regulators | CLGN | calmegin | 0.3 |

| Category | Gene Symbol | Title | AVG Fold Change |
|---|---|---|---|
| miscellaneous | BTN3A3 | butyrophilin, subfamily 3, member A3 | 0.3 |
| metabolism | FAH | fumarylacetoacetate hydrolase | 0.3 |
| signal transduction | DUSP2 | dual specificity phosphatase 2 | 0.3 |
| miscellaneous | --- | H. sapiens transcr sequence, moderate simil. to prot. ref:NP_054848.1 | 0.3 |
| cytoskeleton, structural proteins | COBL | KIAA0633 protein | 0.3 |
| signal transduction | NDP | Norrie disease (pseudoglioma) | 0.3 |
| DNA/RNA proteins, transcription factors | EIF2S3 | eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa | 0.2 |
| growth factors, hormones, cytokines | IGF1 | insulin-like growth factor 1 | 0.2 |
| transporter, carrier proteins | SNAP23 | synaptosomal-associated protein, 23kDa | 0.2 |
| immunomodulators | B7-H4 | immune costimulatory protein B7-H4 | 0.2 |
| immunomodulators | GW112 | differentially expressed in hematopoietic lineages | 0.2 |
| ECM/cell adhesion | CTNNA2 | catenin alpha 2 | 0.2 |
| miscellaneous | CGI-38 | brain specific protein | 0.2 |
| signal transduction | FKBP5 | FK506 binding protein 5 | 0.1 |
| metabolism | GSTT1 | glutathione S-transferase theta 1 | 0.1 |
| immunomodulators | SCGB1D2 | secretoglobin, family 1D, member 2 | 0.1 |
| matrix metalloproteinases | MMP26 | matrix metalloproteinase 26 | 0.1 |
| proteases, peptidases | SERPINA3 | serine (or cysteine) proteinase inhibitor, clade A, member 3 | 0.1 |
| cytoskeleton, structural proteins | TNNC1 | troponin C, slow | 0.0 |

# Dankwoord

Dit proefschrift is het resultaat van de inzet van velen. Tientallen uren zijn besteed aan het bedenken en verfijnen van de onderzoeksvragen, honderden endometriumbiopten en menstruumsamples zijn verzameld en verwerkt, duizenden CAMs zijn getransplanteerd, tienduizenden coupes zijn gesneden en gekleurd, en ontelbare uren zijn gespendeerd om alle vergaarde informatie te verwerken. Heel veel dank aan iedereen die zich voor dit boekje heeft ingezet!

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# Curriculum Vitae

De auteur van dit proefschrift werd op 18 maart 1973 geboren in Zetten. Na het doorlopen van de lagere school in Zetten en het Stedelijk Gymnasium in Nijmegen werkte zij als au pair in Basel, Zwitserland. In 1992 werd de studie geneeskunde begonnen aan de Katholieke Universiteit Nijmegen. Tijdens haar studie liep ze een onderzoeksstage in Asin Foso, Ghana (onderzoek: "*Home management of diarrhoea in under-fives in rural Ghana*") en een tropencoschap in Sumve, Tanzania. Het artsexamen werd op 15 juni 1999 behaald (cum laude). Van juni tot en met december 1999 werkte zij als AGNIO Gynaecologie en Verloskunde in ziekenhuis Rijnstate in Arnhem. Van januari 2000 tot en met december 2003 was ze werkzaam als IVF-arts en onderzoeker op de IVF-afdeling van het academisch ziekenhuis Maastricht. In 2000 hield ze zich bezig met onderzoek naar afwijkingen in de bloedstolling bij vrouwen met pre-eclampsie in de voorgeschiedenis en bij vrouwen die IVF ondergingen. Vanaf 2001 maakte ze deel uit van de endometrium-onderzoeksgroep onder leiding van dr. G.A.J. Dunselman, dr. P.G. Groothuis, dr. A.F.P.M. de Goey en prof. dr. J.L.H. Evers en werd begonnen met de studies beschreven in dit proefschrift. Het onderzoek werd uitgevoerd binnen de afdeling ontwikkelingsbiologie van het onderzoeksinstituut Groei en Ontwikkeling (GROW) van de Universiteit Maastricht. In 2003 ontving ze de Pélérinprijs voor artsassistenten werkzaam in het azM voor de voordracht: "*Remming van angiogenese voorkomt vorming van endometriose-achtige laesies in het chorio-allantoïs membraan (CAM) model*". In 2004 won zij voor haar presentatie: "*Angiostatic therapy for endometriosis: a mouse study*" de Promising Young Scientist award tijdens de 20th Annual Meeting of ESHRE in Berlijn. Vanaf 1 januari 2004 is ze gynaecoloog in opleiding in het academisch ziekenhuis Maastricht (opleider: prof.dr. J.L.H. Evers). Annemiek is getrouwd met Peter Krijns en moeder van Jasper.

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